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Presented by:	Diplom-Biologin Iva Nesic
Born in:	Banjaluka

**Analysis of γ -secretase regulation by
phosphatidylethanolamine using mammalian and
Drosophila melanogaster in vitro and *in vivo* model
systems**

Referees: Prof. Dr. Dr. h.c. Konrad Beyreuther
Prof. Dr. Werner Buselmaier

Ich widme diese Arbeit meinen Eltern.

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Abbreviations

A	alanine
Aβ	amyloid β peptide
AD	Alzheimer's disease
AICD	APP intracellular domain
APP	amyloid precursor protein
BACE	β -site APP cleaving enzyme
CEPT	CDP-ethanolamine phosphotransferase
Ct	carboxy terminus
CTF	carboxy terminal fragment
D	asparagine
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides
DTT	dithiothreitol
DRM	detergent resistant microdomains
EDTA	ethylenediaminetetraacetate
ER	endoplasmic reticulum
ETNK	ethanolamine kinase
FAD	familial early-onset AD
FBS	fetal bovine serum
G	glycine
GFP	green fluorescent protein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
K	lysine
kb	kilobase
kDa	kilodalton
Leu	leucine
Lys	lysine
M	methionine
N	asparagine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PECer	phosphoethanolamine ceramide
PECT	phosphoethanolamine cytidyltransferase
PI	phosphatidylinositol
PL	phosphoglycerolipids
PS	presenilin / phosphatidylserine
RNA	ribonucleic acid
RNAi	RNA interference
SDS	sodium dodecyl sulphate
TLC	thin layer chromatography
V	valine

Summary

A β -amyloid peptide (A β), that plays a central role in the pathogenesis of Alzheimer's disease is derived by sequential proteolytic processing from the amyloid precursor protein (APP) by β - and γ -secretase. APP is additionally processed through a non-amyloidogenic pathway by α -secretase. Recent work suggests that amyloidogenesis is highly dependent on the levels of cholesterol within plasma membrane/early endosomes' microdomains termed "rafts". Indeed APP cleaving machinery, required for A β generation has been shown to reside in lipid rafts and the secretase activity on APP to depend on membrane cholesterol levels. Counterintuitive to the localization of cleavage machinery, the substratum protein APP localizes, at constitutive levels of expression, in membrane microdomains enriched in phospholipids (PL), so-called non-raft domains. From these two series of results it arises that not only cholesterol-rich rafts but also cholesterol-poor/PL-rich non-rafts could be important modulators of AD implicated APP processing. In this work, I have addressed the question of how changes in the lipidic content of non-raft domains, where APP concentrates, affect proteolytic processing of this protein. As phosphatidylethanolamine (PE), an important regulator of diverse cell processes, accounts for the majority of PL I focused on the regulation of APP proteolysis by this particular PL. For this purpose I utilized *Drosophila melanogaster* and mammalian model systems. Confirming previous work, APP was found in the non-raft domains of either insect or mammalian cells, excluded from cholesterol/ergosterol/Flotilin that enrich in rafts. The activity of γ -secretase on APP that is the crucial step in A β generation was assayed in *Drosophila in vivo* system using fly strains transgenic for human APP-C-terminal fragment, fused to the GAL4-VP16 (GV) transcription factor. Membrane PE levels in these γ -secretase reporter flies were depleted by introducing the eas^{PC80} mutation, that affects ethanolamine kinase (ETNK) an enzyme involved in PE synthesis pathway. A strong downregulation of hAPP-Ct processing by γ -secretase, readout by GV triggered cell lethal GRIM and GFP reporter genes expression was observed in low membrane PE flies compared to the γ -reporter flies with wild-type membrane PE levels. The effect of PE on APP proteolysis was additionally observed in mammalian HEK 293 cells stably expressing hAPP. In these cells membrane PE levels were altered by the treatment with RNAi directed against diverse PE synthesis enzymes including ETNK. Membrane PE level decrease, caused by RNAi treatment was shown to correlate with a downregulated γ - and β -secretase processing of APP and correspondingly an elevated α - secretase activity on APP. In the present study I could show that besides cholesterol/raft microdomains, PL (in particular PE), which are the major lipids in APP surrounding non-raft microdomains, appear to be involved in the regulation of APP proteolysis. From all the above I conclude that APP cleavage efficiency is highly dependent on the levels of the lipidic environment of non-raft domains, either because of affecting the degree of accessibility of the responsible cleaving enzymes to APP or by affecting the capacity of these enzymes to cleave the substratum. These are in my view important venues for future investigation, opened by this work.

Zusammenfassung

Das Amyloid β Peptid, das eine zentrale Rolle in der Pathogenese von Alzheimer Erkrankung (AD) spielt, entsteht durch die sequentielle Prozessierung des Amyloid Precursor Protein (APP), durch die β - und γ -Sekretase. Zusätzlich kann APP in einem nicht-amyloiden Prozessierungsweg, durch die α -Sekretase innerhalb der β -Domäne proteolytisch gespalten werden. Aktuelle Studien deuten darauf hin, dass die Amyloidogenese in hohem Maße von dem Gehalt an Cholesterol in Mikrodomänen innerhalb der Plasmamembran bzw. früher Endosomen abhängt. Diese cholesterolreiche Membrandomänen werden als "Rafts" bezeichnet. Es ist gezeigt worden, dass die APP-Prozessierungsmaschinerie, welche für die Amyloidogenese benötigt wird, in Rafts angereichert ist, und dass die Aktivität der Sekretasen von dem Cholesterolgehalt in der Membran abhängt. Anders als seine Prozessierungsmaschinerie, ist APP unter physiologischen Bedingungen hauptsächlich in den Membrandomänen lokalisiert, die reich an Phospholipiden (PL) sind und als "Non-Rafts" bezeichnet werden. Diese beiden Erkenntnisse führen zu der Annahme, dass nicht nur cholesterolreiche, sondern auch die cholesteroolarme/PL-reiche Membran-Mikrodomänen eine entscheidende Rolle in der Regulierung der APP-Prozessierung und der Entstehung des pathogenen β -Peptids spielen.

In der vorliegenden Arbeit habe ich die Frage untersucht, ob und wie die Veränderungen in der Lipidkomposition der Non-Raft-Domänen, wo APP konzentriert ist, dessen proteolytische Prozessierung beeinflussen. Da Phosphatidylethanolamin (PE) zu den Hauptbestandteilen von Non-Raft-Mikrodomänen zählt, wurde im Rahmen dieser Arbeit die Regulierung der APP-Prozessierung durch dieses spezielle PL eingehend untersucht. Zu diesem Zweck wurden humane Zellen und *Drosophila melanogaster* als experimentelle Systeme verwendet. In Übereinstimmung mit vorangegangenen Studien, wurde APP sowohl in humanen Zellen als auch in Insektenzellen in den PL-reichen, Non-Raft-Domänen gefunden, in den keine Raft-typischen Lipide (Cholesterol/Ergosterol) und Proteine (Flotillin) angereichert waren. Für die Untersuchung der APP-Proteolyse durch die γ -Sekretase, die den entscheidenden Schritt in der β -Peptid Entstehung darstellt, wurde das *Drosophila in vivo* System verwendet, genauer, transgene Reporterstämme, die das humane APP-C-terminale Fragment exprimieren, das mit GAL4-VP16, einer Transaktivator-domäne, fusioniert ist. GAL4-VP16 aktiviert die Transkription der Reportergene, deren Expression ein direkter Indikator der γ -Sekretase-Aktivität ist. Die Reportergene, die γ -Sekretase Aktivität indizieren, sind GRIM, das die Apoptose in der Retina induziert und das Gen für das "Green Fluorescent Protein", GFP. Der Membrangehalt von PE in γ -Sekretase-Reporterfliegen wurde durch die Kreuzung mit den *eas*^{PC80}-Mutanten reduziert. Die *eas*^{PC80}-Mutation hat einen direkten Einfluss auf das Enzym Ethanolamin Kinase, das für die PE-Synthese verantwortlich ist.

Vergleiche zwischen den γ -Sekretase-Reporterfliegen, die einen Wild-typ PE-Membranlevel aufweisen und den Reporterfliegen, mit dem niedrigen PE-Gehalt, zeigen in den letzteren eine starke Reduktion der γ -Sekretase-Aktivität, die durch das Ausmaß des retinalen Zelltodes und der GFP-Intensität indiziert wird. Zusätzlich konnte ein Effekt von Membran-PE auf die APP-Proteolyse in den humanen HEK-Zellen, die das humane APP exprimieren, festgestellt werden. Der Membrangehalt von PE wurde in diesen Zellen durch den RNAi vermittelten knock-down der PE-Syntheseeenzyme signifikant beeinflusst. Die Reduktion des Membran-PE-Gehalts, durch die RNAi-Behandlung, zeigte eine klare Korrelation mit der starken Reduktion von β - und γ -Sekretase-Aktivitäten. Die Prozessierung von APP durch die α -Sekretase war stark erhöht. In der hier vorliegenden Studie konnte ich zeigen, dass neben Cholesterol und den Raft Mikrodomänen, den PL (hierbei besonders PE), den meist vertretenen Lipid-Spezies, in den APP angereicherten Non-Rafts, eine wichtige Rolle in seiner Proteolyse zukommt. Die oben genannten Gründe lassen deshalb den Schluss zu, dass die APP-Prozessierung stark von der Beschaffenheit der Non-Raft-Domänen abhängig ist. Die Rolle von Non-Rafts in der APP-Regulierung kann darauf beruhen, dass sie den Zugang der Sekretasen zu dem Substrat modulieren oder direkt die Aktivität, der für die APP Prozessierung verantwortlichen Enzyme beeinflussen. Diese Arbeit deutet darauf hin, dass die Non-Rafts ein wesentliches Stellglied für die APP-Prozessierung sind und eröffnet damit neue Einblicke in die Regulationsmechanismen, den die APP-Prozessierung und viel wichtiger, die Amyloidogenese, unterliegen.

1. Introduction

1.1. Alzheimer's Disease (AD)

Alzheimer's disease, a progressive neurodegenerative disorder of the central nervous system has been called "the disease of the century" because of its staggering medical and social dimensions. On November 4th, 1906 Alois Alzheimer first published his observations on the rapidly deteriorating mental illness in a 51-year old woman called Auguste D, who had shown progressive cognitive impairment, focal symptoms, hallucinations, delusions, and psychosocial incompetence. Characteristic clinical features of AD besides a wide range of cognitive dysfunctions, of which the most common is the failure of memory for recent events (Terry et al., 1994, Cummings 2000), are impairments in attention, anxiety and emotional modulation (Ferretti et al., 2001, Foldi et al., 2002). These conditions give a rise to a range of symptoms like depression, panic, sleep disturbances, paranoia and delusion. Alzheimer's is the most common form of dementia. Epidemiological studies show that AD affects 5-10% of the population over 65, 20% after age of 40 and 43% after age of 90 years. Most aggressive forms of AD, caused by autosomal dominant inheritance of certain mutations, may have an onset younger than 30 years.

Histological analyses from the brains of demented patients versus non-demented patients revealed the presence of bundles of fibrils within the neurons and numerous focal lesions in the cerebral cortex. Most affected are the brain regions responsible for cognitive function like, cerebral cortex, entorhinal cortex and hippocampus. The brains of patients suffering AD are characterized by extracellular accumulation of amyloid β peptide ($A\beta$) and intracellular aggregates of hyperphosphorylated tau, in so-called amyloid plaques and neurofibrillary tangles respectively. Although not the exclusive pathological signs these are the major hallmarks of AD.

Accumulation of aggregated $A\beta$, as the key pathological event driving neurodegeneration in AD and Down's syndrome was introduced by George Glenner (Glenner and Wong, 1984). Sequential cleavage of APP, by its cleaving enzyme termed γ -secretase generates 38-43-amino-acid $A\beta$ peptides (Haas and

De Strooper, 1999). The longer A β peptide (A β 42), which represents 10% of all A β species in the brain, seems to be the more amyloidogenic form of the peptide since it is prone to aggregate. Hence A β 42 peptides have an increased propensity to accumulate as extracellular amyloid deposits in senile plaques and cerebral blood vessels. Together with shorter fragments of the peptide in amorphous form they constitute senile plaques in AD brains (Selkoe, 1999). Additionally to the 7-10nm-thick amyloid fibrils the mature plaques contain degenerating axons and dendrites, and are surrounded and invaded by variable numbers of microglia and reactive astrocytes.

Neurofibrillary tangles are intracellular aggregates of hyperphosphorylated tau (Lee et al., 2001) consisting of 10nm-thick paired helical filaments. Aggregate formation relies on elevated level of phosphorylated tau in the cells (Grundke-Iqbal et al., 1986). Tau, a microtubule-associated protein is phosphorylated by protein kinases, such as the neuron-specific cyclin dependent kinase 5 (cdk5). This event precedes the organisation of highly phosphorylated tau protein in paired helical fragments that causes the disruption of microtubules and ultimately leads to cell death (Cruz et al., 2003, Noble et al., 2003). Together with the tau containing dystrophic neurons, neurofibrillary tangles are likely to be a consequence of A β peptide accumulation (Hardy and Selkoe, 2002).

Although the pathology of AD is complex, amyloid cascade hypothesis, which states that chronic imbalance between A β production and clearance leads to a multistep cascade including gliosis, inflammatory changes, neuritic/synaptic change, tangles and transmitter loss is considered to be the primary cause for dementia in AD pathogenesis (Scheuner et al. 1996, Price et al., 1998, Hardy and Selkoe, 2002, St. George-Hyslop and Petit, 2005, Tanzi and Bertam, 2005). A β peptide is not produced as an independent protein but is instead generated by proteolytic processing of a type I transmembrane protein, the amyloid precursor protein (APP) (Kang et al., 1987, Tanzi et al., 1987). The role of APP in Alzheimer's disease became interesting as the mutations in the *APP* gene, localized on chromosome 21 have been found in some rare forms of familial early-onset AD (FAD) (Chartier-Harlin et al., 1991, Goate et al., 1991, Murrell et

al., 1991, Eckman et al., 1997). Most of *APP* missense mutations alter APP processing in a pathological manner by increasing either overall production of A β peptide or generating highly fibrillogenic A β variants, like A β 42. (Citron et al., 1992, Cai et al., 1993, Haas et al., 1994, Suzuki et al., 1994, Price et al., 1998). Interestingly AD related APP mutations were found within the region encoding A β or immediately adjacent to β - and γ -secretase cleavage sites (Hardy, 1996) that strengthen the amyloid cascade hypothesis. In *APP* mutations linked FAD that accounts for less than 5% of total AD cases, clinical and pathological symptoms are nearly identical to that of the more frequent, late-onset sporadic AD. The observations above strongly suggest that abnormal processing of APP plays an important role not only in FAD but also in sporadic form of AD.

In addition to mutations in *APP* gene, FAD cases were linked to the mutations in genes encoding presenilin-1 (PS1) and presenilin-2 (PS2), localized on chromosome 14 and 1, respectively (Sherrington et al., 1995, Rogaev et al., 1995, Levy-Lahad et al., 1995). Biochemically, FAD-associated mutations in *PS* gene increase the relative concentration of the aggregation-prone A β 42 (Sisodia, 2002, Iwatsubo, 2004). The mechanisms by which these mutations lead to a selective increase of in the levels of A β 42 species have not been fully resolved yet.

There is also strong evidence that the ϵ 4 allele of apolipoprotein E (ApoE) is associated with increased AD risk (Roses, 1998). ApoE4 plays a crucial role in the metabolism and clearance of A β peptide. Like *APP* and *PS* genes, ApoE ϵ 4 allele impacts on A β production, deposition or clearance. This provides strong genetic support to the role of amyloid cascade hypothesis in AD.

1.2. Amyloid Precursor Protein (APP)

Senile plaques in the brains of AD patients are deposits composed primarily of β -amyloid insoluble peptides generated from the Amyloid Precursor Protein (APP). APP is known to play a central role in the pathogenesis of AD but its

physiological function is still not fully understood. Experiments in *Drosophila melanogaster* indicate that APP might play an important role in the regulation of cell adhesion and signalling (Beyreuther and Masters, 2001). Besides its role in AD development A β domain appears to be crucial for correct APP functioning in processes like axonal adhesion, neuritic outgrowth or regulation of synaptic plasticity.

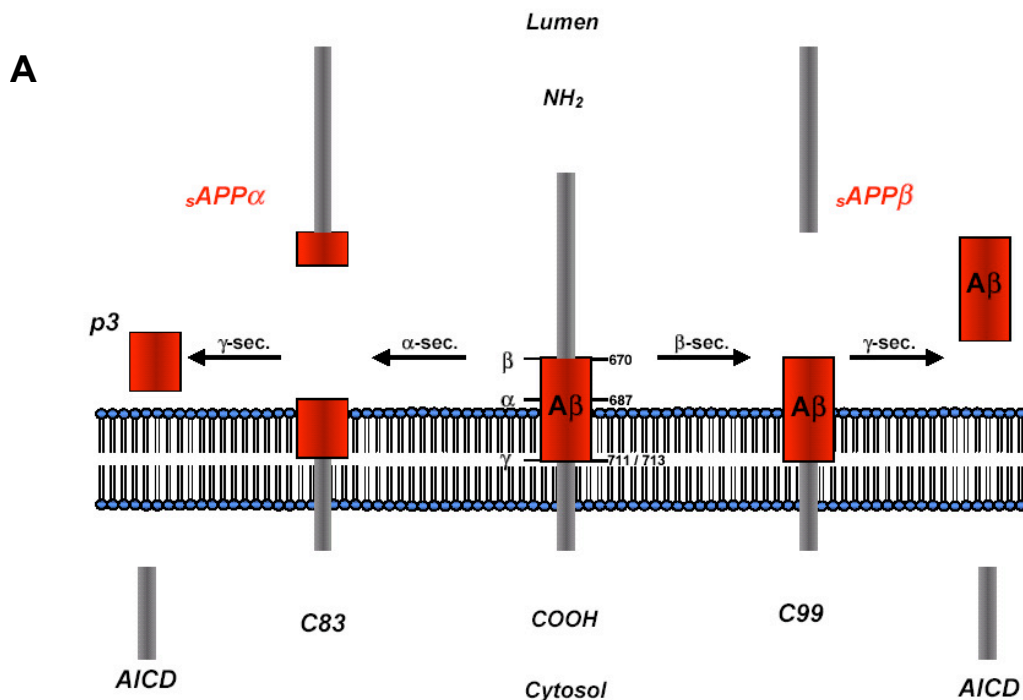
APP is a 110-120-kDa integral type I membrane glycoprotein that contains a large amino terminal extracellular/cytosolic domain and a small intracellular COOH-terminal domain (De Strooper and Annaert, 2000, Nunan and Small, 2000). This ubiquitously expressed protein is modified in the secretory pathway by N-glycosylation and O-glycosylation in the endoplasmic reticulum (ER) and the Golgi apparatus (Weideman et al., 1989, Sinha and Lieberburg, 1999). Tyrosine sulfation and addition of phosphate in trans-Golgi network and at the cell surface further increase the structural complexity of this protein. APP is expressed as three alternatively spliced isoforms: APP695 (neuronal form), APP770 and APP771 (peripheral and glial isoforms). APP770 and APP771 contain Kunz-type protease inhibitor domain within the ectodomain.

Mammalian APP belongs to a protein family with two other members: the amyloid precursor like protein 1 and 2 (APLP1 and APLP2). Two homologues have been identified in invertebrates: the amyloid protein-like protein 1 (APL-1) in *Caenorhabditis elegans* (Daigle, 1993) and the amyloid precursor protein-like protein (APPL) in *Drosophila melanogaster* (Rosen, 1989). None of the homologous members of the APP protein family exhibit sequence similarities within the β -amyloid region that encodes the AD implicated A β peptide (Selkoe, 1996).

1.2.1. Proteolytic processing of APP

APP proteolytic processing is a membrane related event, which occurs by sequential cleavage of APP by proteases termed α -, β - and γ -secretase (Figure 1.1.). This process involves ectodomain shedding by either α - or by β -secretase

and the retention of corresponding membrane-anchored C-terminal fragment, α CTF (C83) and β CTF(C99). γ -secretase a multimeric protein complex subsequently cleaves the α and β CTFs, in the middle of transmembrane domain generating a 3-kDa peptide (p3) and A β (4-kDa), respectively. Additionally to p3 or A β generation, γ -cleavage releases the intracellular domain of APP (AICD) after C83 or C99 processing. The APP processing pathway, in which α -secretase precedes the γ -secretase cleavage is so-called non-amyloidogenic pathway and is the favoured pathway in non-neuronal tissue. The production of β -amyloid peptide, relevant for AD pathogenesis is the product of sequential APP cleavage by β - and γ -secretase and it is the major APP processing pathway in neurons. Both amyloidogenic and non-amyloidogenic pathways occur under physiological conditions which indicates, that all products of APP proteolytic processing, including A β are part of normal physiology (Haass et al., 1992, Seubert et al., 1992).



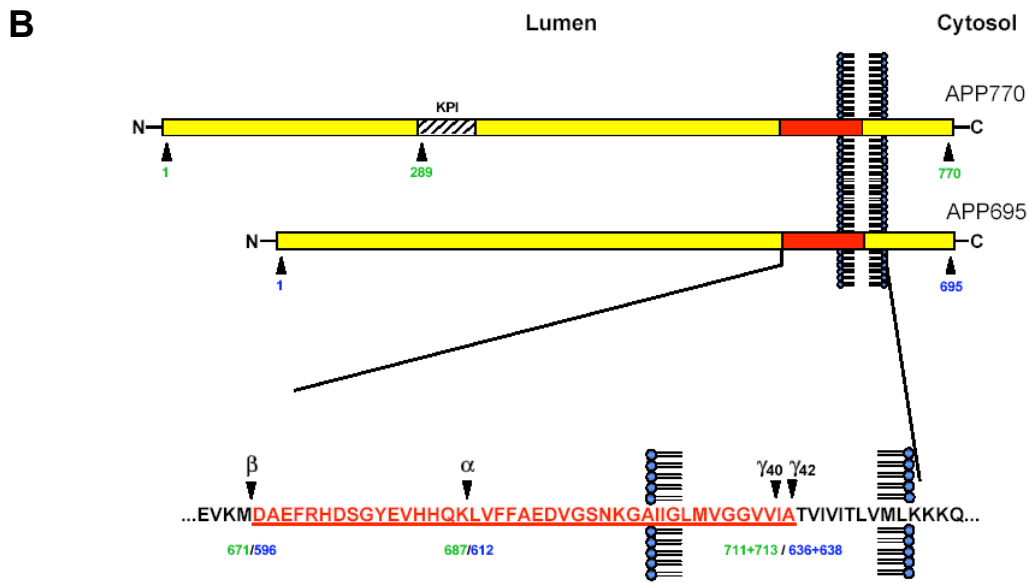


Figure 1.1. (A) Schematic diagram of the proteolytic processing of amyloid precursor protein (APP). APP is a type I transmembrane protein that is processed by α -, β - and γ -secretase. APP processing by α -secretase is characterized by the release of a large soluble ectodomain fragment (sAPP α) and the retention of a 10-kDa membrane anchored fragment (C83). α -secretase cleaves APP within the A β domain (marked in red) and prevents amyloidogenesis. Extracellular β -secretase mediated cleavage produces a soluble APP- β fragment (sAPP β) that is shed in the extracellular space and a membrane attached C-terminal fragment (C99). The membrane bound carboxy-terminal fragments C99 and C83 are immediate substrates for γ -secretase that cleaves in the middle of transmembrane domain and generates 4kDa A β peptide and a small p3 peptide, respectively. Additionally γ -secretase cut releases the APP intracellular domain (AICD) that is known to be involved in nuclear signalling. **Figure (B) depicts schematic diagram of APP770 and APP695 isoforms and amino acid sequences of the regions encompassing the A β sequence and the intracellular domain.** The APP770 isoform contains a serine protease inhibitor domain of the Kunz type (KPI, starting from the residue 289), while APP695 lacks this domain and is therefore shorter than APP770. The amyloid- β sequence (labelled in red) is identical in both isoforms. Protease cleavage sites in APP770 isoform (marked in green) are as follows: β -secretase cleaves after residue 671, α -secretase cleaves after residue 687 and γ -secretase cleaves after residues 711 and 713, generating A β 40 and A β 42, respectively. APP695 isoform is

cleaved by proteases at following cleavage sites (marked in blue): β -secretase cleaves after residue 596, α -secretase cleaves after residue 612 and γ -secretase cleaves after residues 636 and 638, generating A β 40 and A β 42, respectively.

α -secretase cleaves APP within A β domain between residues Lys16 and Leu17 (numbering according to the primary sequence of A β peptide), and therefore precludes generation of intact A β peptide. As already mentioned above during APP cleavage by α -secretase, a soluble ectodomain (sAPP α) is released into extracellular space and a 10-kDa C-terminal fragment remains within the membrane (C83) (Weidemann et al., 1989). SAPP α has been shown to have neurotrophic properties (Small 1998), which could antagonize the neurotoxic effect of A β peptide. Cleavage of C83 by γ -secretase releases a short peptide (p3), containing the C-terminal part of A β peptide. The biological significance and the role of p3 in amyloidogenesis remain obscure. Several zinc metalloproteases including TACE (tumor necrosis factor- α converting enzyme; or ADAM17), ADAM (a disintegrin and metalloprotease) 9, ADAM10 and MDC9 and an aspartyl protease BACE2 can cleave APP at the α -secretase site (Allinson, 2003).

The APP cleavage by α -secretase may occur in the *trans*-Golgi compartment (Sinha and Lieberburg, 1999), at the cell surface or within calveolae (Lammich et al., 1999, Kosik, 1999). At the membrane level α -secretase that is involved in non-amyloidogenic APP processing pathway has been shown to act in the non-raft membrane microdomains (Kojro et al., 2001, Ehehalt et al., 2003). ADAMs are not only found in vertebrates but also in *Caenorhabditis elegans*, *Drosophila melanogaster* and *Xenopus*. It has been shown that *Drosophila* α -secretase processes human APP in a similar manner like in mammalian cells, generating a CTF corresponding in size to mammalian α CTF (C83) (Fossgreen et al., 1998, Loefer et al., 2003). One of the best candidates responsible for this α -secretase activity in *Drosophila* is the protease Kuzbanian (Kuz). Kuz belongs to the ADAM family, members of which contain both a disintegrin and metalloprotease domain.

β -secretase is involved in amyloidogenic processing of APP and is the first prerequisite for generation of A β peptide. This enzyme cleaves APP at the Asp+1 residue of A β sequence, and generates two products: a secreted soluble fragment (sAPP β) and the membrane bound APP C-terminal fragment, composed of 99 amino acids (β CTF or C99). Subsequently γ -secretase cleaves C99 producing a spectrum of intact β -amyloid peptide plus the APP intracellular domain (AICD). A β -site APP cleaving enzyme, BACE (beta-site APP-cleaving-enzyme; also called Asp-2 and memapsin-2) has been identified by several groups by genetic screening, and by direct enzyme purification and sequencing (Hussain et al., 1999, Vassar et al., 1999, Yan et al., 1999). BACE is a transmembrane protein characterized by a large extracellular domain, containing two aspartate residues involved in β -secretase activity (Hussain et al., 1999) and it is a member of pepsin family of aspartyl proteases. Affinity of BACE1, which is the major neuronal β -secretase toward APP is relatively low. Interestingly, *Swedish* type of missense mutation in *APP* gene, which is known to enhance A β generation, promotes cleavage of APP by BACE (Vassar et al., 1999).

β -secretase has maximal activity at acidic pH (Haass et al., 1993 and 1995, Knops et al., 1995). Indeed cell biological studies indicate that BACE colocalizes with endosomes and *trans*-Golgi (Koo and Squazzo, 1994, Haass et al., 1995, Walter et al., 2001) and that its activity is highest in these subcellular compartments that provide acidic environment. Another important aspect regarding β -secretase activity is its localization and activity on APP within the lipid rafts (Ridell et al., 2001, Eehalt et al. 2003, Cordy et al., 2003, Abad-Rodriguez et al., 2004). Homologues for BACE in invertebrates, e.g. in *Drosophila melanogaster* have not been reported.

γ -secretase cleavage of APP is the major step in the generation of A β peptide. This intramembrane protease cleaves APP fragments generated by α - and β -secretase within the membrane and produces secreted fragments consisting of

nontoxic p3 and toxic β -amyloid peptide, respectively and the intracellular domain of APP, AICD. As described above γ -cleavage of APP C-terminal fragments generates a spectrum of amyloidogenic species and the exact position of γ cleavage site is critical for AD pathogenesis. Besides APP γ -secretase cleaves a number of substrates like E-cadherin, N-cadherin, ErbB-4, CD44, p75 neurotrophin receptor, and most importantly the protein Notch (Struhl and Greenwald, 1999, 2001, Ye et al., 1999, Thinakaran and Parent, 2004), which is involved in cell fate decision in the embryo and in the adult. The process in which γ -secretase cleaves APP and Notch within the plasma membrane is an example of a much more general process known as regulated intra-membrane proteolysis (RIP). RIP is a highly conserved from bacteria to humans and it is involved in a numerous cellular regulatory events, including lipid homeostasis (Brown et al., 2000).

γ -secretase is a membrane-bound enzyme complex comprised of at least four components including Presenilin 1 and 2 (PS1, PS2), Nicastrin (Nct), anterior pharynx-defective phenotype (APH-1) and Presenilin enhancer (PEN-2) (Figure 1.2.). Several studies indicate that γ -secretase endoproteolyzes its substrates through a GX'GDX''-type catalytic site (X' is variable, and X'' is preferably a hydrophobic amino acid) residing within the 6th and the 7th transmembrane domain (TMD6, TMD7) of PS (Steiner et al., 2000). Hence Presenilins appear to be a central catalytic component of γ -secretase. **Presenilin (PS)** is an aspartyl protease whose membrane topology is characterized by 7- to 8-membrane spanning domains (Figure 1.2.), with the sixth and seventh domain containing aspartate residues (Asp257 and Asp385), responsible for the catalytic activity. PS is synthesized as an immature, inactive holoprotein, which is subsequently endoproteolyzed by an unknown activity to generate N- (NTF, ~30kDa) and C-terminal (CTF, ~20-kDa) fragments, which are thought to associate to form the active enzyme (Thinakaran et al., 1996, Rattovitski et al., 1997, Levitan et al., 2001). **Nicastrin (Nct)**, a glycosylated type I transmembrane protein (~130-kDa) contains a large N-terminal extracellular and a short C-terminal intracellular

domain (Figure 1.2.). Nct is an additional putative component of γ -secretase complex, which exists, similar to PS as an immature and a mature protein (Edbauer et al., 2002, Leem et al., 2002). The mature isoform of Nct is associated with active γ -complex. N-terminal part of Nicastrin, that contains several conserved cysteines and a conserved functionally important DYIGS motif, associates with PS and COOH-terminal fragments of APP and Notch (Yu et al., 2000, Chen et al., 2001, Capell et al., 2003) and it has been proposed to be required for PS-mediated cleavage at the cell surface in *Drosophila* (Chung and Struhl, 2001) and mammalian cells (Kaether et al., 2002). But PS and Nic alone do not suffice for the formation of an active γ -secretase complex. Indeed two other members of γ -secretase complex were identified through genetic screens in *Caenorhabditis elegans*, APH-1 and PEN-2. **APH-1** is a 30-kDa multipass membrane protein containing seven transmembrane segments. Together with Nct APH-1 works as a scaffold and facilitates the assembly and trafficking of γ -secretase (La Voie et al., 2003, Niimura et al., 2005). **PEN-2** a 12-kDa membrane protein with two transmembrane segments is incorporated into the PS-Nct-APH-1 complex through direct binding to TMD4 of PS. It causes PS endoproteolysis, which avails its proteolytic activity to other substrates (Takasugi et al., 2003, Watanabe et al., 2005, Kim et al., 2005).

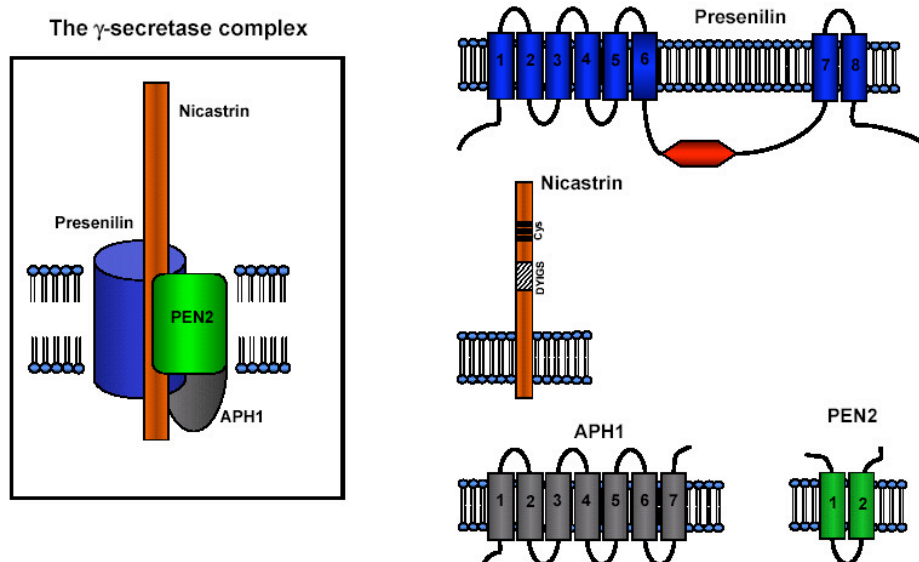


Figure 1.2. Schematic depiction of the γ -secretase complex and its putative components: Presenilin (PS), Nicastrin (Nct), APH-1 and PEN-2. γ -secretase is a membrane bound protein complex consisting of at least four different integral membrane proteins: PS, Nic, APH-1 and PEN-2 (left panel). PS contains eight transmembrane domains, NH₂- and COOH-terminal cytoplasmic domains, and a large intracellular hydrophilic loop domain (right panel). Catalytic aspartate residues in PS transmembrane domains 6 and 7 indicate that PS may comprise the active site of γ -secretase. Endoproteolytic processing of immature PS generates a PS heterodimer, which is thought to be component of the mature, active γ -secretase. The red box represents the endoproteolytic cleavage site. Nct is a type I transmembrane protein, with an extracellular domain that contains several conserved cysteines and a functionally important DYIGS motif, and a relatively small intracellular domain. Together with APH-1 and PEN-2, that contain seven and two transmembrane segments, respectively, Nct is required for the maturation, trafficking and the activity of the γ -secretase complex.

At the subcellular level, γ -secretase activity have been shown to reside in multiple compartments including the ER, late-Golgi/TGN, endosomes and the plasma membrane (Xu et al., 1997, Cook et al., 1997, Greenfield et al., 1999, Takahashi et al., 2002, Kaether et al., 2002). Only 6% of total γ -activity had been detected at the cell-surface (Chyung et al., 2005). The majority of the active components of γ -complex are present in ER/Golgi intermediate compartments, Golgi apparatus, the trans Golgi network (TGN), and late endosomes (Vetrivel et al., 2004). Recent biological evidence suggests that γ -secretase components assemble into the proteolytically active complex in the Golgi/TGN compartments (Baulac et al., 2003). Similar to BACE1, active components of γ -secretase activity, namely PS1 derived fragments, mature Nct, APH-1 and PEN-2 associate with lipid raft microdomains (Vetrivel et al., 2004), although interestingly γ -secretase activity seem to be independent of the presence of cholesterol (Wada et al., 2003). Presenilins, Nicastrin, APH-1 and PEN-2 enable the highly conserved γ -secretase proteolytic activity in mammalian, insect or yeast cells (Takasugi et al., 2003, Kimberly et al., 2003, Hayashi et al., 2004).

A novel APP cleavage taking place a few residues C-terminal to the γ -site has been recently reported (Gu et al., 2001, Yu et al., 2001, Sastre et al., 2001, Weidemann et al., 2002). Remarkably, it shares similarities with S3 Notch cleavage site. The **ϵ -cleavage** site of APP resides at Leu-49 distal to the γ -secretase site, and is thought to be involved in the regulation of APP cleavage generating APP intracellular domain, that similar to its Notch counterpart appears to mediate important physiological functions. Evin and colleagues suggest that ϵ -CTF may be derived from γ CTF by a rapid action of a second protease in the cytosol (Weidemann et al., 2002). Alternatively ϵ -cleavage may represent an intermediate step in A β formation that would precede γ -cleavage at 40- or 42-position (Weidemann et al., 2002). This observation indicates that γ - and ϵ -cleavages on APP could be seen as either deleterious (γ -site) or beneficial (ϵ -site) events, and that modulation of ϵ -cleavage may contribute to the regulation of the neurotoxic amyloid- β peptide generation implicated in AD pathogenesis. This cellular mechanism is still poorly understood and further studies are required to clarify the function of this cleavage.

1.2.2. Modulators of APP proteolytic processing

Generation of amyloid- β peptide by APP proteolytic processing plays a central role in the pathophysiology of AD (Beyreuther and Masters, 1997) and is therefore an interesting target from pharmaceutical point of view. Particularly modulation of APP by β - and γ -secretase, enzymes involved in amyloidogenic processing of APP may stop or prevent neurodegenerative processes characteristic for AD. But also α -secretase cleavage of APP is an issue in amyloidogenesis, as α -secretase cleaves APP within A β domain preventing the deposition of intact A β peptide. Moreover sAPP α , a large soluble domain, produced by α -processing of APP has neuroprotective and memory-enhancing effects (Barger and Harmon, 1997, Meziane et al., 1998).

As already described APP and its proteolytic machinery consisting of α -, β - and γ -secretase reside at the membrane. It seems likely that APP and the proteases being membrane proteins respond to their lipid environment in a way that could eventually affect their activity. Indeed strong evidence indicates a functional relationship between AD and amyloidogenesis with lipid metabolism (Simons et al., 2001, Kalvodova et al., 2005, Vetrivel and Thinakaran, 2006). Although alterations in several lipid species including phospholipids, ceramides and sphingolipids have been reported to correlate with AD pathogenesis and AD related events (Prasad et al., 1997, Han, 2005, Kalvodova et al., 2005), cholesterol and cholesterol rich membrane domains, rafts have received the most attention during the past few years (Puglielli et al., 2003, Wolozin, 2004, Vetrivel and Thinakaran, 2006).

The reasons for the interest in this lipid are several fold. There are epidemiologic data that show a direct relationship between cholesterol and AD. For instance high cholesterol levels are shown to be an important risk factor in AD pathogenesis (Hofman et al., 1997). Further, Kivipelto et al. (2001) reported that elevated cholesterol levels during mid-life increase the risk of developing the disease. Additionally cholesterol metabolism is genetically linked to AD through ApoE ϵ 4 allele (Sing and Davignon, 1985), which is the major genetic factor for developing the disease (Strittmatter et al., 1993, Corder et al., 1993). Clinical studies indicating that there is a decreased prevalence of AD associated with the treatment of cholesterol synthesis inhibitors (statins) (Jick et al., 2000, Wolozin et al., 2000, Austen et al., 2002) are supported by studies in animal models that have revealed a significant decrease of A β generation in animals fed with statins (Fassbender et al., 2001) and on the other hand an accumulation of A β due to the cholesterol rich diet (Sparks et al., 1996, Refolo et al., 2000). The epidemiological data find support in cell biological studies. Thus, cholesterol reduction at the cellular level by inhibition of its *de novo* synthesis with statins alone or in combination with cholesterol extracting agent, methyl- β -cyclodextrin results in a strong decrease of A β production (Simons et al., 1998, Fassbender et al., 2001, Ehehalt et al., 2003). Amyloidogenic processing of APP by γ - and β -

secretase is affected by cholesterol depletion as indicated by a marked reduction of β CTF and A β peptide generation (Simons et al., 1998, Ehehalt et al., 2003). The non-amyloidogenic APP processing pathway seems to be stimulated by cholesterol loss, since reduction of cholesterol levels causes an increase in the production of APP ectodomain generated by α -secretase (Kojro et al., 2001). Taken together the results described above suggest that cholesterol depletion favours the non-amyloidogenic pathway, while inhibiting the AD implicated amyloidogenic APP processing by β - and γ -secretase.

Contrary to the above view it has been shown that mild reduction of cholesterol leads to an increased amyloidogenesis in neurons indicating the importance and requirement of cholesterol for APP proteolysis (Rodriguez et al., 2004). These results are supported by observations showing that rodents treated with the statin most permeable to the blood brain barrier show an increased A β generation and senile plaque deposition (Park et al., 2003). The discrepancy between these last results with the above may rely on several factors (see Kaether and Haass, 2004, Ledesma and Dotti 2006) and future work is needed to settle this controversy. Nevertheless what is clear from all these two series of results is that cholesterol can play a key role in the regulation of APP processing, possibly by restricting the access of proteases to their substrate. (Kaether and Haass, 2004, Kalvodova et al., 2005). Hence an altered A β peptide generation in cholesterol low membranes is likely to rely on an aberrant access of β - and γ -secretase to their substrate. A crucial cell membrane structure that may act as a link between cholesterol and APP processing are cholesterol/sphingolipid enriched lateral assemblies within the membrane so-called lipid rafts.

Biological membranes are complex structures composed of lipids, that have multiple and distinct roles in cellular function (Roberts et al., 2002, Hardie et al., 2003). Although membranes contain thousands of individual lipid molecular species three lipid species are the main components of cellular membranes: sterols, sphingolipids and phosphoglycerolipids (phospholipids) (Figure 1.3.).

Sterols are based on a rigid four-ring structure, with cholesterol being the principle form found in vertebrate. Sphingolipids contain C18 alcohol sphingosine or dihydrosphingosine, their homologs or their derivatives (ceramide) associated with either a phosphocholine headgroup (sphingomyelin) or one of a range of carbohydrate structures (glycosphingolipids). In sphingolipids both acyl chains are often saturated. Phosphoglycerolipids comprise one of several headgroups (phosphocholine, phosphoethanolamine, phosphoserine and phosphoinositol) attached via glycerol to two acyl chains, one of which is usually unsaturated. Sphingolipids are longer and more saturated than phospholipids, and would therefore be predicted to have a higher melting temperature than phospholipids.

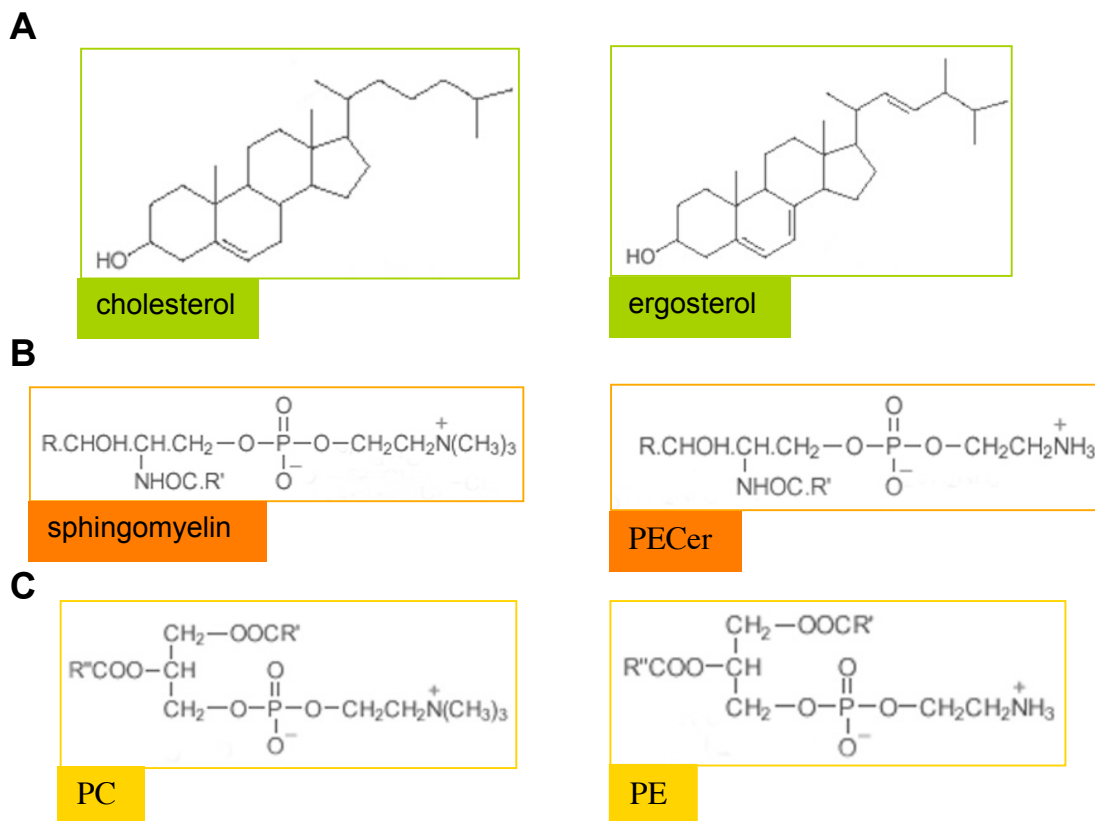


Figure 1.3. Major membrane lipid species in mammals and *Drosophila*. (A) Sterol structure is based on a rigid four-ring structure. Cholesterol is the most abundant sterol in the membranes of the mammalian cells. Flies cannot synthesize sterols and require therefore a dietary source that is mainly yeast with ergosterol being the major sterol

species. **(B)** The structural unit of mammalian and *Drosophila* sphingolipids is C18 alcohol sphingosine or dihydrosphingosine, their homologues or their derivatives (ceramide). Sphingomyelin, the major sphingolipid in mammalian membranes is composed of ceramide and phosphocholine. Instead in *Drosophila* membranes phosphoethanolamine ceramide (PECer) accounts for the majority of membrane sphingolipids and it contains ceramide and phosphoethanolamine. Typically, amide-linked fatty acids (R') present in sphingolipids are saturated or monounsaturated. **(C)** Most phosphoglycerolipids (phospholipids) contain a saturated fatty acid (R') on C-1 and an unsaturated fatty acid (R'') on C-2 of the glycerol backbone. The most commonly added alcohols (serine, ethanolamine and choline) also contain nitrogen that may be positively charged, whereas, glycerol and inositol do not. The major phospholipid species in mammals besides phosphatidylcholine (PC) is phosphatidylethanolamine (PE) that is present in *Drosophila* membranes with 55% of total phospholipids.

At the subcellular levels sterols and sphingolipids are not distributed homogeneously (Ikonen, Vainio and Lusa, 2005). Other than in the plasma membrane, the membranes of early endosomes and the endosomal recycling compartment, which are enriched in sterols and sphingolipids, these lipids are present at low levels in endoplasmic reticulum (ER) and cis Golgi membranes. Also within membranes lipid distribution is not homogenous. Instead membrane lipids have high affinities towards each other and tend to form lateral assemblies within the membrane. One such assembly termed rafts are known to be an important regulator of polarized intracellular sorting and signal transduction (Simons and Ikonen, 1997, Brown and London, 1997, Simons and Toomre, 2000). Moreover a large body of evidence suggests that these specialized membrane domains are related to AD pathogenesis (see above, and Simons et al., 1998, Simons et al., 2001, Fassbender et al., 2001, Eehalt et al., 2003, Abad-Rodriguez et al., 2004, Vetrivel and Thinakaran, 2006). Lipid raft formation is based on the tendency of cholesterol to organize the membrane bilayer in cholesterol-rich liquid ordered and cholesterol-poor liquid disordered domains (Sankaram and Thompson, 1991). Pure phospholipid bilayers can exist in two states a "gel" state or "liquid" state. The presence of rigid sterol in the

physiologically relevant liquid phase causes phospholipid acyl chains to become closely packed or compacted, and the bilayer to be thickened. This high cholesterol bilayer in which phospholipid acyl chains cannot readily deform is termed liquid-ordered phase, in contrast to liquid-disordered state “without” cholesterol. Formation of liquid-ordered phase is enhanced by preferential interaction of cholesterol with sphingolipids and the fact that sphingolipids have a higher melting temperature than phospholipids. The “separation” of cholesterol/sphingolipid-rich liquid-ordered membrane domains from the phospholipid-rich phase that constitutes the rest of the membrane leads to raft formation. Raft assembly takes place in the Golgi, and they are predominantly located at the plasma membrane. Biochemically lipid rafts are defined as detergent resistant microdomains (DRM) that resist extraction with a non-ionic detergent, like Triton X-100 (Yu et al., 1973, Brown and Rose, 1992, London and Brown, 2000, Edidin, 2003).

Raft microdomains have also been found in the membranes of *Drosophila melanogaster* (Rietveld et al., 1999). *Drosophila* membranes are similar to those in mammals regarding their lipid composition. Like in mammalian membranes major lipidic components in *Drosophila* membranes are sterols, sphingolipids and phosphoglycerolipids. In contrast to mammalian membranes in which cholesterol accounts for the majority of membrane sterols, in *Drosophila* membranes ergosterol represents the major sterol species (Figure 1.3. A). Flies incorporate dietary (yeast) sterols into their membranes, as they have only a part of sterol synthesis machinery and are therefore disabled to synthesize sterols. The only phosphosphingolipid in mammals, sphingomyelin is not present in *Drosophila* membranes. Instead *Drosophila* membranes contain phosphoethanolamine ceramide (PECer) (Figure 1.3. B). Despite differences in chemical structure between mammalian and *Drosophila* lipids, the properties of sterols, sphingolipids and phospholipids that allow raft formation have been preserved. Both ergosterol and PECer are enriched in insoluble membranes (Rietveld et al., 1999).

As mentioned above, strong evidence indicates that rafts may be the principal membrane platforms where amyloidogenic processing of APP takes place. The importance of raft microdomains for APP processing, particularly in amyloidogenic pathway is strongly supported by the enrichment of entire A β generating machinery within the lipid raft microdomains (Li et al., 2000, Wahrle et al., 2002, Ehehalt et al., 2003, Rodriguez et al., 2004, Vetrivel et al., 2004). A further evidence for the role of rafts in amyloidogenesis is the concentration of monomeric and oligomeric A β in DRM in the brains of a mouse model for AD (Lee et al., 1998, Kawarabayashi et al., 2004). Opposite to β and γ -secretase activity in rafts, α -secretase involved in non-amyloidogenic APP processing takes place outside rafts (Ehehalt et al., 2003).

On the other hand, it is also strong the evidence that amyloidogenic processing of APP, is mostly taking place within phospholipid rich or non-rafts membrane domains (Rodriguez et al., 2004). This would be consistent with the view of Ehehalt et al. (2003), who suggest the existence of APP in two pools, in raft and non-raft domains, rendering possible that both, lipid rafts and non-raft domains are important regulators of APP cleavage. Although large body of evidence support the cholesterol/raft role in APP processing event, very little is known about the role of APP surrounding phospholipidslipids and non-rafts in its proteolytic processing. The colocalization of APP with these membrane domains strongly suggests the phospholipid role as APP cleavage modulators and the analysis of their role in APP proteolysis may shed important light on amyloidogenesis.

Although phospholipids are major components of cell membranes, the role of phospholipids in the physiology of the cell is just beginning to be understood. Phospholipids play multiple roles in cells being important components in cell signalling, by providing the matrix for the assembly and function of a wide variety of catalytic processes and therefore actively influencing the functional properties of membrane-associated processes (Berridge and Irvine 1984, Nishizuka 1986, Fadok et al., 1992, 2000, Vance and Vance, 1996, Dowhan et al., 1997, Alessenko and Burlakova, 2002). One of those membrane related events

implicated in AD (BACE-activity) has been shown recently by Simons and colleagues to be regulated by phospholipids (Kalvodova et al., 2005). Similar to the sterols phospholipids show altered levels in the brains of Alzheimer's patient. In particular PE membrane levels are affected in AD brains while PC and phosphatidylinositol (PI) do not show significant changes in AD brains compared to control (Wells et al., 1995, Prasad et al., 1997). Among all phospholipids PE appears to be the most interesting candidate for modulation of APP proteolytic processing. Besides PC, PE is the most abundant phospholipid in eukaryotic cells, and it constitutes 20-40% and 55% of the total phospholipids in mammalian and in *Drosophila* cell membranes, respectively (Figure 1.3. C). PE is involved in a variety of cell processes. It has been shown to be required for membrane protein activity, it serves as "chaperone" and, along with other "non-bilayer" lipids, PE seems to be important for maintaining the physical state of the bilayer (Bogdanov et al, 1996, van der Brink-van der Laan, 2004). Recently *Drosophila* PE has been shown to regulate SREBP (sterol regulatory element-binding protein) processing, an event that is regulated by sterols in mammalian cells (Dobrosotskaya et al., 2002). Unlike PC and PS, which form flat bilayers, PE can form non-bilayer structures under physiological conditions. These structures, observed *in vitro*, may provide discontinuity in the membrane bilayer for several important biological functions like vesicle-mediated protein trafficking, lateral movement of macromolecules within bilayer, stabilization of specific membrane protein complexes (Dowhan et al., 1997).

PE is synthesized via two main pathways: CDP-ethanolamine pathway (Kennedy et al., 1957) and phosphatidylserine (PS) decarboxylation pathway (Merrill, 1997, Dickson and Lester, 1999, Hannun and Luberto 2001; Figure 1.4.). While in insects the CDP-ethanolamine pathway is known to be the major source of PE (Downer et al., 1985), in mammalian cells the utilization of that pathway is tissue dependent. Kennedy pathway is the primary pathway in mammalian tissues such as the brain and the heart (Butler and Morell, 1983, McMaster et al., 1993, Arthur and Page, 1991). In liver and kidney tissue PE is mainly synthesized by PS-decarboxylation.

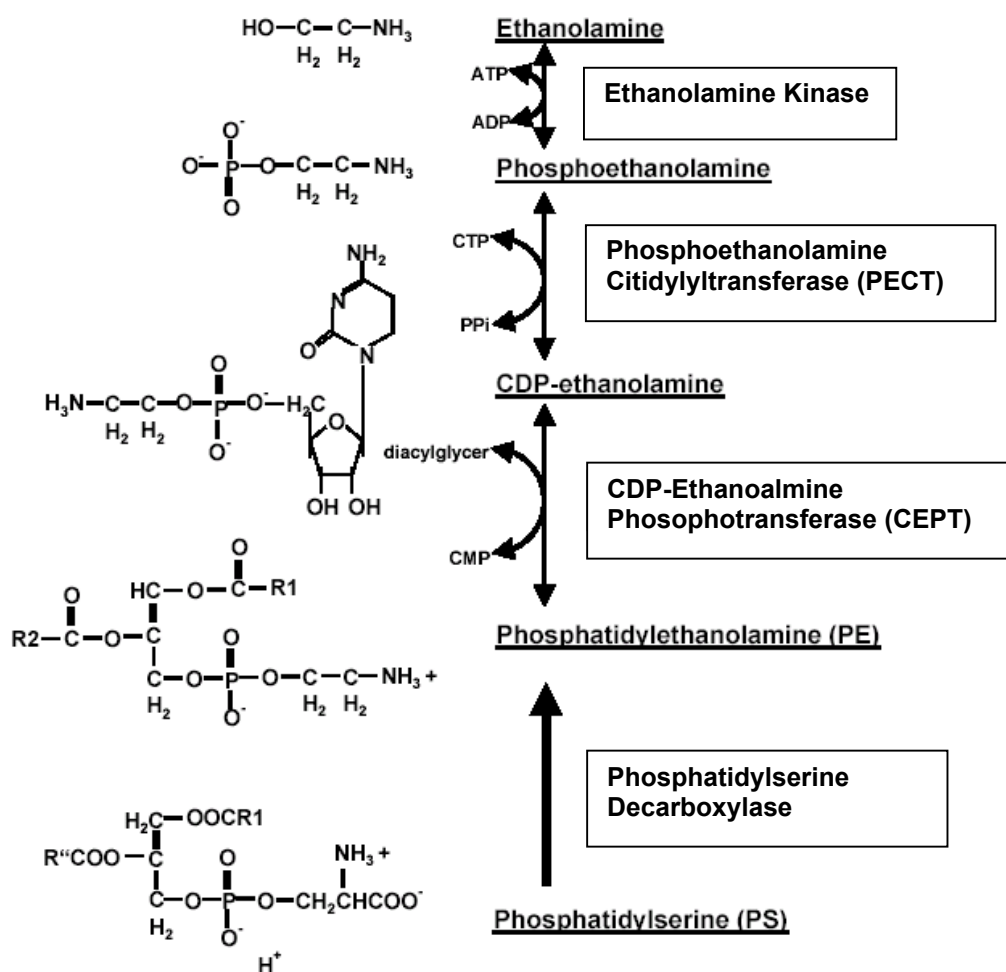


Figure 1.4. The major pathways for biosynthesis of phosphatidylethanolamine *de novo* in eukaryotic cells. Scheme is based on data from mammalian and yeast systems. PE is mainly synthesized through CDP-ethanolamine pathway and phosphatidylserine decarboxylation pathway. The first step in CDP-ethanolamine pathway which is the major pathway in *Drosophila* and in mammalian neuronal tissue is phosphorylation of ethanolamine by ethanolamine kinase (ETNK), followed by reaction of the product with cytidine triphosphate (CTP) to form cytidine diphosphoethanolamine (CDP-ethanolamine). CDP-ethanolamine phosphotransferase, catalyses the reaction of the last compound with diacylglycerol to form phosphatidylethanolamine (PE). Additionally to CDP-ethanolamine pathway, PE is synthesized through decarboxylation of phosphatidylserine by phosphatidylserine decarboxylase.

1.3. Goals of my PhD thesis

The processing of APP by its cleaving enzymes plays a crucial role in AD pathogenesis, and is therefore an important pharmacological target. Membrane lipids in particular cholesterol/sphingolipid rich membrane microdomains are important candidates for regulation of APP proteolysis. The localization of APP processing enzymes responsible for amyloidogenic APP cleavage supports the regulatory role of cholesterol and rafts in amyloidogenesis. But the finding that APP localization in neurons and non-neuronal cells is largely restricted to the phospholipid rich liquid disordered phase strongly indicates the importance of these membrane domains for APP processing and amyloidogenesis. In addition, the crucial role that PE plays in variety of membrane occurring enzymatic activities strengthen the hypothesis that PE may be an important factor in the regulation of APP proteolysis. Present study has been performed in order to understand whether PE plays a modulatory role in the regulation of APP proteolytic processing by α -, β - and γ -secretase, as indicated by several factors described above.

The modulation of the APP processing by PE will be analysed by utilizing *Drosophila in vivo* and *in vitro* model system and mammalian *in vitro* model system. In the first part of my thesis I will focus on the analysis of PE role on α -, β - and γ -secretase activity by using mammalian HEK 293 cells, overexpressing wild-type APP.

The second part, in which the experiments will be performed in a more sophisticated *Drosophila in vivo* system is aimed at addressing the PE modulatory role on the crucial step of A β generation, namely APP processing by γ -secretase. Presenilin dependant γ -secretase activity, that is highly conserved in mammals and *Drosophila melanogaster* will be analysed in the flies transgenic for APP-C-terminal fragment, initiating just downstream of the β -secretase cleaving site that is known to be processed in *Drosophila* in a similar manner like in mammalian cells, generating A β peptide and APP intracellular domain (AICD).

Transgenic fly lines utilized for that purpose GMR-APP-GAL4, UAS-GRIM and APP-GAL4-VP16, UAS-cd8-GFP act as a sensitive and specific reporter of the endogenous, physiological levels of γ -secretase. PE levels in transgenic flies will be modulated by introducing the *eas*^{PC80} mutation with a known biochemical effect in phospholipid metabolism, particularly in PE synthesis that allows the investigation of specific consequences of defects in phospholipids, or rather PE metabolism. The properties of *Drosophila* sterols, sphingolipids and phospholipids that allow the formation of liquid ordered/raft phase and liquid disordered/non-raft phase provide optimal conditions to analyse the role of membrane microdomains in APP proteolysis. Additionally I will analyse the influence of membrane PE on γ -secretase activity in *Drosophila* embryonic Schneider-2 (S2) cells.

2. Materials and Methods

2.1. Cell biology

2.1.1. HEK 293-hAPP cells

2.1.1.1. Culturing HEK 293-hAPP cells

Human epithelial kidney 293 cells (HEK 293), stably transfected with wild-type APP695 (the cells were kindly provided by Dr. Christian Haass) were grown in Doelbeco's Minimal Essential Medium (DMEM-Gibco) containing 10% fetal bovine serum (FBS, Sigma), 2 mM L-glutamine (Sigma), 100u/ml Penicillin Streptomycin solution (Sigma), 200 µg/ml G418 sulfate antibiotic Liquid (Invitrogen) at 37°C and 5% CO₂.

HEK 293 cells stably transfected with the APP cDNA containing the *Swedish* mutation were generous gift from Dr. De Strooper. The cells were maintained in DMEM with 10% FBS, 100u/ml Penicillin Streptomycin solution and 250 µg/ml G418 at 37°C and 5% CO₂.

2.1.1.2. RNAi transfection of HEK 293-hAPP cells

On day before transfection, HEK 293-hAPP cells were harvested by trypsinization and 6×10^5 cells were plated in 4 ml of growth medium without antibiotics per 6 cm dish. At the time of transfection cells were 50% confluent. Prior to transfection cells were washed once in Opti-MEM I Reduced Serum Medium (Invitrogen) and subsequently switched to 3 ml of the same medium of per dish. 1000 pmol RNAi were diluted in 500 µl of Opti-MEM I Reduced Serum Medium for each sample. 10 µl Lipofectamine 2000 (Invitrogen) were diluted in 500 µl of Opti-MEM I Reduced Serum Medium, and after 5 minutes incubation at room temperature combined with the diluted RNAi. RNAi/Lipofectamine mixture was incubated for 15 minutes at room temperature and subsequently added to the cells. RNAi transfected cells were incubated at 37°C and 5% CO₂. Cells were assayed 48 hours after RNAi transfection.

2.1.2. *Drosophila* Schneider-2 (S2) cells

2.1.2.1. Culturing S2 cells

S2 cells grow at 25⁰C without CO₂ as a loose, semi-adherent monolayer. The cells were purchased from Invitrogen and grown in Schneider's Insect Medium (Sigma) containing 10% heat-inactivated FBS (Sigma) and 100u/ml Penicillin Streptomycin solution.

2.1.2.2. DNA transfection of S2 cells

Schneider cells were transiently transfected with 15 µg of SC100 construct that contains hC99 fragment (Maryama et al., 1994; Figure 2.1.), with Calcium Phosphate Transfection Kit (Invitrogen). At day 1, 3 x 10⁶ cells were seeded in 3ml of S2 complete growth medium in each well of a 6-well format and incubated overnight at 25⁰C without CO₂. At day 2, 3-4 hours before transfection cells were switched to the fresh complete growth medium. Transfection mixture was prepared according to the manufacturer's protocol and added to the cells after 30 minutes of incubation at room temperature. 18 hours post-transfection calcium phosphate solution was removed, and the cells washed twice with 1 x Phosphate Buffered Saline (PBS). Subsequently S2 cells were resuspended in 3 ml fresh complete growth medium and replated to the same vessel. Cells were incubated at 25⁰C without CO₂ and analysed for the gene of interest 48 or 72 hours after transfection.



Figure 2.1. Amino acid sequence encoding for APP695-C-terminal fragment (hC99; M596-N695) that is contained in the SC100 construct. The fragment initiates just C-terminal to the β -secretase cleavage site (M596) and encompasses the A β domain and the intracellular domain. The cleavage sites of α -, β - and γ -secretase are indicated by arrowheads. A β domain is shown in red.

2.1.2.3. RNAi transfection of S2 cells

S2 cells were set up for the RNAi transfection by placing 2×10^6 cells in 2 ml of antibiotic free S2 growth medium into each well of 6-well culture dish. After 24 hours cells were resuspended in 1.5 ml of serum and antibiotic free IPL-41 Insect Medium (Invitrogen). Subsequently 50 μ g/ml of RNAi were added to the cells. Six hours later, 1.5 ml of IPL-41 Insect Medium containing 20% of FBS were added to each well, and the cells were incubated over night at 25°C without CO₂. S2 cells were re-suspended, re-treated with RNAi and re-plated each 24 hours and analyzed 48 hours after the first RNAi treatment.

2.1.3. Determination of cell viability

Cell viability was analysed by measuring apoptosis and necrosis level. Apoptosis was scored by using Apoptosis Detection System, Fluorescein (Promega). This assay is based on DNA labelling by catalytically incorporating of fluorescein –12-

dUTP^(a) at 3'-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT), which forms a polymeric tail using the principle of the TUNEL (TdT-mediated dUTP Nick-End labelling) assay. The cells were prepared for the assay as described in manufacturer's protocol and analyzed by fluorescence microscopy. Necrosis was measured by Trypan blue uptake. Trypan blue (Sigma) stock solution of 8% was prepared in 0.9% NaCl, diluted 1:9 in HBSS and subsequently mixed 1:1 with the cell suspension. Cell viability is indicated by the fraction of dye-excluding cells.

2.2. Biochemistry

2.2.1. Preparation of cell lysates for protein and lipid analysis

HEK 293-hAPP cells were washed once in 1ml of cold washing buffer (1 x PBS, CLAP (chymostatin, leupeptin, antipain and pepstatin A, each of the final concentration of 25µg/ml, Sigma)), mechanically removed from the culture dishes in 2 ml of lysis buffer (1 x PBS, CLAP, 0.2% Triton X-100 (Merck)), incubated for 15 minutes on ice and homogenized by 10 passages through a 27-gauge syringe.

The medium of S2 cells was collected and the cells that grow in suspension were centrifuged to pellet for 5 minutes by 1000 x g at 4⁰C (Microcentrifuge 5415R, Eppendorf). Cell pellet was washed in 1 x PBS/CLAP, resuspended in lysis buffer containing 1 x PBS, CLAP and 0.2% (v/v) Triton X-100, and subsequently incubated for 15 minutes on ice in order to avoid the protein degradation. After 15 minutes on ice cells were homogenized by 10 passages through a 27-gauge syringe. HEK 293-hAPP and S2 cell lysates were centrifuged for 10 minutes by 400 x g at 4⁰C to pellet the nuclei and to remove cell debris; the supernatants were considered as total extracts. Protein concentration was assayed in Spectrophotometer Genesys 10 UV-VIS (Thermo Spectronic), using Bradford reagent (Bio-Rad Laboratories).

2.2.2. DRM separation using Triton X-100

HEK 293-hAPP or S2 cells were extracted in a buffer containing 25 mM MES (2 (N-morpholino)-ethanesulfonic acid) pH 7.0, 5 mM DTT, 2 mM EDTA pH 8.0, 1% Triton X-100, CLAP. After 1 hour of incubation in lysis buffer at 4°C under rotation, cells were centrifuged 10 minutes by 400 x g at 4°C in order to pellet the nuclei and to remove cell debris. Membrane pellets were obtained by cell extracts (containing 100 µg of total protein) centrifugation for 1 hour at 4°C at 100, 000 x g in Ultracentrifuge Optima Max E (MLA-103 rotor; Beckman). Extracted membrane pellets were resuspended in 1 ml of solution buffer (10 mM MES, 2mM EDTA, 1mM DTT), brought to 40% OptiPrep™ solution (AXIS-SHIELD PoC AS) and overlaid with 30% and 5% OptiPrep™ solution in solution buffer. The subsequent OptiPrep™ step gradient centrifugation was performed in Ultracentrifuge Le80 (SW40 rotor; Beckman) at 120, 000 x g for 18 hours at 4°C. After centrifugation 1 ml of each of following gradient fractions were collected: 5 % fraction, the interphase between 5% and 30% fraction, 30% fraction and 40% fraction. Subsequently each fraction was centrifuged by 100, 000 x g for 1 hour at 4°C to extract the membranes that were utilized for the analysis of membrane lipids and membrane proteins. The interphase between the fractions 5% and 30% was identified as the DRM fraction by the presence of the DRM markers.

2.2.3. Western blot analysis

For analysis of membrane proteins total cell extracts were centrifuged for 1 hour at 100, 000 x g at 4°C to pellet the membrane fraction. Crude membranes were resuspended in 0.2% (w/v) Sodium Dodecyl Sulfate (SDS) and after protein measurement by Bradford protein assay the proteins were denatured for 5 minutes in 1 x SDS sample buffer (22mM Tris-HCl pH 6.8, 0.8% (w/v) SDS, 4% glycerol, 1.6% (w/v) β-Mecaptoethanol, Bromphenolblue) at 95°C. Samples for the analysis of membrane bound proteins isolated from the membranes fractionated by OptiPrep gradient were prepared as described above. Cytosolic

proteins were directly diluted in 1 x SDS sample buffer and denatured for 5 minutes at 95°C. Denatured proteins were subjected either to the Tris-glycine-acrylamide SDS-PAGE or to the urea version of the Bicine/bis-Tris/Tris/sulfate SDS-PAGE (Wiltfang et al., 1991) and separated by electrophoresis. Tris-glycine-acrylamide gels were run at constant voltage of 100 volts, and the urea version of the Bicine/bis-Tris/Tris/sulfate SDS-PAGE gels were run at room temperature at the constant current of 12 mA/gel, using the Mini-PROTEAN II electrophoresis cell (Bio-Rad Laboratories).

After gel electrophoresis, proteins were transferred to a 0.45 µm nitrocellulose transfer membrane (Schleicher & Schuell BioScience) using a constant voltage of 100 volts for 1 hour, with transfer buffer (48 mM Tris, 38 mM glycine, 0.037% (v/v) SDS, 20% (v/v) methanol) in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories). Subsequently membrane was incubated in blocking solution (5% (w/v) non-fat powder milk dissolved 1 x in TBS-T buffer (25 mM Tris/HCl pH 7.4, 150 mM NaCl, 4 mM KCl, 0.1% (v/v) Tween-20) at 4°C overnight. Primary and secondary antibodies were diluted in blocking solution containing 1% (w/v) powder milk to the appropriate concentrations (Table 1). Species specific peroxidase-conjugated antibodies and the enhanced chemiluminescence (ECL) (Amersham Biosciences) were used followed by the exposure to x-ray films (BioMax Light Film for Chemiluminescent Imaging, Kodak Co). Quantification was done by densitometry of autoradiograms using Image J software.

Primary antibody	Description	Clonality	Company	Dilutions for WB
ANTI-AMYLOID PRECURSOR PROTEIN (APP) C-Terminal	C-Terminus (95-100 kDa) of APP695, APP770 and APP771	polyclonal	Sigma	1:1000
Monoclonal Anti-Human β Amyloid [1-17] Clone 6E10	Residues 1-17 of the human β -amyloid peptide	monoclonal	Sigma	1:500
A.v. Peptide Antibody	Central and C-Terminal portion of <i>Aequorea victoria</i> green fluorescent protein	monoclonal	BD Biosciences	1:400
Anti-Flotillin-1	Flotillin-1 ~48k-Da	monoclonal	BD Biosciences	1:500
Anti- α -Tubulin	α -Tubulin ~60-k-Da	monoclonal	Calbiochem	1:1000
E18	A β amino terminal-selective	monoclonal	nanoTools	1:300
WO2	A β residues 5-8	monoclonal	Abeta GmbH	1:1000

Secondary antibodies	Company	Dilution
Horse radish peroxidase antibody (sheep anti mouse)	Amersham	1:5000
Horse radish peroxidase antibody (donkey anti rabbit)	Amersham	1:5000

Table 1 Collection of primary and secondary antibodies that were used for Western blot (WB) assay.

2.2.4. Immunoprecipitation of A β peptides and their electrophoretic separation.

For analysis of A β peptide, medium from the S2 cells was collected and immunoprecipitated. Magnetic sheep anti-mouse immunoglobulin G (IgG)

Dynabeads M-280 (Dyna) were preactivated by overnight incubation at 4°C with monoclonal A β amino terminal-selective antibody, 1E8 (nanoTools) according to the manufacturer's protocol. Eight hundred μ l of medium were added to 200 μ l fivefold concentrated RIPA detergent buffer (2.5% (v/v) Nonidet-P40, 1.25% (w/v) sodium deoxycholate, 0.25% (v/v) SDS, 750 mM NaCl, 250 mM HEPES, one tablet of Protease Inhibitor Cocktail Complete per 2ml of 5 x RIPA, pH 7.4), 25 μ l preactivated magnetic Dynabeads (1 ml 1E8 mouse antibody/ 1.68×10^7 beads) and 300 μ l H₂O. Samples were then incubated overnight at 4°C under rotation, following washing of the beads four times with PBS/0.1% bovine serum albumin, and once with 10 mM Tris/HCl, pH 7.4. For A β -SDS-PAGE-Immunoblot, bound A β peptides were eluted by heating the samples to 95°C for 5 minutes with 25 μ l sample buffer. For separation of A β peptides the urea version of the Bicine/bis-Tris/Tris/sulfate SDS-PAGE was used. Gels were run at room temperature for 2 hours at the constant current of 12 mA/gel, using the Mini-PROTEAN II electrophoresis cell (Bio-Rad Laboratories). A β peptides were transferred for 30 minutes at 1 mA/cm² and room temperature under semidry Western conditions onto Immobilon-P PVDF membranes (Millipore) (according to Witlfang et al., 1997) and incubated in the blocking solution containing 2.5% (w/v) non-fat powder milk 1 hour at room temperature. The membranes were then immunostained overnight with monoclonal amino terminal-selective antibody, 1E8 (nanoTools) at 4°C. After the washing step the membranes were incubated for 1 hour at room temperature with an anti-mouse biotinylated antibody (Vector Laboratories), washed, and horseradish peroxidase-coupled streptavidin (Amersham Biosciences) was added for 1 hour. After the final washing step, chemiluminescence was visualized with ECLPlus solution (Amersham Pharmacia) according to the protocol of the manufacturer, using the CCD camera system (FluorSMaxMultimager, Bio-Rad).

2.2.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA assay, utilized for A β 42 detection in the extracellular medium from the HEK 293-SweAPP695 cells, was performed using the highly sensitive microtiter plate Enzyme-Immunoassay for the quantitative detection of human Amyloid β 42, hAmyloid β 42 ELISA (HS) (the GENETICS company). This assay is based on the formation of an antibody-Amyloid-antibody complex, which is indirectly linked to an enzyme that catalyses the conversion of the substrate in a coloured product which colour intensity is measured by means of photometry. The measured extinction correlates directly with the concentration of hA β 42 within the sample. The analysis was done according to the manufacturer's protocol.

2.2.6. Analysis of soluble APP α in the medium of HEK 293-hAPP cells

Soluble APP α , which is released into the medium after APP cleavage by α -secretase was analysed in the medium of HEK 293-hAPP cells. For sAPP α analysis 50 μ l medium were diluted in 50 μ l 2 x sample buffer and heated to 95 $^{\circ}$ C for 5 minutes. The samples were subjected to 10% SDS-PAGE and run at the room temperature at the constant voltage of 200 volts for 40 minutes, using the Mini-PROTEAN II electrophoresis cell (Bio-Rad Laboratories). After electrophoresis a Western blotting was performed using semidry conditions and Immobilon-P PVDF membranes. Subsequently the membranes were incubated for 1 hour at room temperature in the blocking solution containing 2.5% (w/v) non-fat powder followed by the overnight antibody incubation with monoclonal amino terminal-selective antibody, 1E8 (nanoTools) at 4 $^{\circ}$ C. After washing with PBS/Tween, incubation at room temperature with an anti-mouse biotinylated antibody (Linaris) and the subsequent incubation with horseradish peroxidase-coupled streptavidin (GE Healthcare) chemiluminescence was visualized with ECLPlus solution (Amersham Pharmacia) using the CCD camera system (FluorSMaxMultimager, Bio-Rad).

2.2.7. Lipid extraction and analysis by Thin Layer Chromatographie (TLC)

Cell membranes obtained after 100, 000 x *g* centrifugation were resuspended in 800 µl of 0.9% (w/v) sodium chloride (NaCl) containing 37% (v/v) chloridc acid (HCl) and homogenized with a 22-gauge syringe. Membrane lipids were than extracted according to Bligh and Dyer (1959). After addition of 2 ml methanol and 1 ml chloroform to 800 µl suspension containing cell membranes, 1 ml chloroform and 1 ml of 0.9% (w/v) NaCl containing 37% (v/v) HCl were added to the sample, mixed well, incubated 10 minutes on ice to allow the phase separation and centrifuged at 1000 rpm for 5 minutes at 4⁰C (Allegra 25R-High-Performing-Centrifuge, TA-14-50 fixed angle rotor; Beckman). The upper phase was discarded and 2 ml methanol plus 1.8 ml of 0.9% (w/v) NaCl/37% (v/v) HCl were added to the interphase and the lower phase and kept on ice for minutes. Subsequently the samples were centrifuged at 1000 rpm for 5 minutes at 4⁰C and the lower phase was collected and dried in a rotational vacuum concentrator (Centrifuge RVC 2-25; Christ) to obtain lipid pellets. Lipid pellets were resuspended in 1:1 methanol/chloroform mixture, applied to the silica gel 60 HPTLC plates (Merck) and separated first in the hydrophilic running solvent, composed of chloroform:acetone:acetic acid:methanol:H₂O (50:20:10:10:5) and subsequently in the hydrophobic running solvent containing hexane:ethyl acetate (5:2). The HPTLC plates were than dried at room temperature and stained with 7% (v/v) sulfuric acid (H₂SO₄) in methanol. Standards for cholesterol, ergosterol and sphingomyeline (Sigma), and PC, PE, and PECeramide (Matreya, Inc.) were used to identify these lipid species. For the lipid quantification the scanned TLCs were analysed in conditions of non-saturated signal by Image J software.

2.3. Molecular Biology

2.3.1. Rapid Small Scale Isolation of *Drosophila* genomic DNA (Walter, 1991)

To prepare genomic DNA from S2 cells, about 20 millions of cells were collected and centrifuged to the pellet at 1000 x *g* for 5 minutes at 4°C. Cell pellet was resuspended in solution A (0.1 M Tris/HCl pH 9.0, 0.1 M EDTA, 1% (v/v) SDS, 1% DEPC, homogenized with a 27-gauge needle and incubated for 30 minutes at 70°C. After incubation 14 µl of 8M KAc per 1 ml of solution A were added and incubated for another 30 minutes on ice. Subsequently the solution was centrifuged for 15 minutes at room temperature, the 1/2 volume of isopropanol was added to the supernatant and centrifuged again for 5 minutes at room temperature. The supernatant was discarded, the pellet was washed with 70% (v/v) ethanol, centrifuged, dried in a speed vacuum concentrator and finally dissolved in 100 µl of pH 7.4 TE buffer (10 mM Tris/HCl pH 7.4, 1 mM EDTA pH 8.0)

2.3.2. RNA interference (RNAi) synthesis

2.3.2.1. RNAi synthesis for the treatment of S2 cells

2.3.2.1.1. Preparation of PCR template for RNAi synthesis directed against *Drosophila* phosphoethanolamine citidyltransferase gene

Two primers, forward and reverse, designed to target *Drosophila* phosphoethanolamine citidyltransferase, PECT (CG5547-gene number refers to the Berkeley *Drosophila* Genome Project designation) messenger RNA have following sequences:

Forward primer 5'-3'

GAATTAATACGACTCACTATAGGGAGAGTGGGCATTACACCGACGAGGA
GATCACC

Reverse primer 5'-3'

GAATTAATACGACTCACTATAGGGAGACTTCAGTGACACAGTAGGGAGCTC
CG

Each primer contains a sequence encoding the T7 RNA polymerase binding site (bold), followed by the appropriate coding sequence. The T7 RNA polymerase binding site sequence is required for the subsequent RNAi synthesis. The oligonucleotides were synthesized by Invitrogen.

The DNA template for RNAi synthesis was synthesized and amplified by PCR reaction (performed in Thermal Cycler PTC 100; MJ research), set-up as follows:

20 µg of *Drosophila* genomic DNA

150 ng of both forward and reverse PECT primers

Taq polymerase buffer (Promega) to final concentration of 1x

dNTPs to final concentration of 0.2 mM each (Promega)

5 units Taq polymerase (Promega)

1.5 mM Mg²⁺ (Promega)

H₂O to a final volume of 100 µl

PCR program

Step 1) 94°C 4 minutes

Step 2) 94°C 15 seconds

Step 3) 58°C 30 seconds

Step 4) 72°C 2 minutes

Step 5) back to the step 2) 30x

Step 6) 72⁰C 7 minutes

The completed PCR reaction was purified using High Pure PCR Product Purification Kit (Roche) according to kit-supplied protocol and examined on a 1% agarose gel.

2.3.2.1.2. Transcription reaction assembly

The Ambion MEGAscript™ T7 kit was used to synthesize RNAi from templates obtained as described under 2.3.2.1.1.

The reaction was set-up as follows:

3 µl H₂O containing 0.1% (v/v) Diethyl pyrocarbonate (DEPC; Sigma)

2 µl 10 x reaction buffer

2 µl of each ATP, CTP, GTP, UTP mixes

5 µl DNA template

2 µl enzyme mix,

and incubated for 8 hours at 37⁰C.

After 8 hours, DNase was added for 15 minutes at 37⁰C. After 15 minutes reaction was stopped with 15 µl of stop solution (5 M ammonium acetate, 100 mM EDTA). The RNAi was extracted by phenol/chloroform extraction method and precipitated with isopropanol overnight at -20⁰C. After precipitation the RNAi was washed with 70% (v/v) ethanol and concentrated in a speed vacuum concentrator. The RNAi was analysed on a 1% agarose gel (Agarose LE; Euroclones) and the concentration was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer using quartz cuvettes. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml ($A_{260}=1=40\text{ }\mu\text{g}$).

The RNAi directed against *Drosophila easily shocked (eas)* gene was designed using Dharmacon siDESIGN Center. The *eas* RNAi and predesigned control RNAi directed against rat CYP7A1 were purchased from Dharmacon RNA TECHNOLOGIES:

<u>easily shocked sense sequence 5'-3':</u>	AAACGGACCUACUGAUAGAUU
<u>easily shocked antisense sequence 5'-3':</u>	UCUAUCAGUAGGUCCGUUUUU
<u>CYP7A1 sense sequence 5'-3':</u>	UAAACAACCUUGACAACUUUU
<u>CYP7A1 antisense sequence 5'-3':</u>	5'-PAAGUUGUCAAGGUUGUUUAUU

2.3.2.2. Synthesis of RNAi oligonucleotides directed against human phosphoethanolamine citidyltransferase, (PECT), CDP-ethanolamine phosphotransferase (CEPT) and ethanolamine kinase 1 and 2 (ETNK1 and ETNK2) genes utilized for the transfection of HEK 293 cells

The RNAi oligonucleotides for the treatment of HEK 293 cells were designed with Dharmacon siDESIGN Center:

<u>ETNK1 sense sequence 5'-3':</u>	CGAUCGAGAUGAGGAAGUA
<u>ETNK1 antisense sequence 5'-3':</u>	UACUUCCUCAUCUGCAUCG
<u>ETNK2 sense sequence 5'-3':</u>	UCAGGUUAAUCGCCUUAGA
<u>ETNK2 antisense sequence 5'-3':</u>	UCUAAGGCGAUUAACCUGA
<u>PECT sense sequence 5'-3':</u>	CCAACAGGUUGGAGUAUGA
<u>PECT antisense sequence 5'-3':</u>	UCAUACUCCAACCGUUGG
<u>CEPT sense sequence 5'-3':</u>	UCAUUGGACUGUCAAUAAA
<u>CEPT antisense sequence 5'-3':</u>	UUUAUUGACAGUCCAUGA

The RNAi oligonucleotides were purchased from MWG Biotech AG.

2.3.3. Propagation and isolation of the SC100 construct

For the transformation with SC100 construct chemocompetent *E.coli* cells (DH5 α strain, Gibco) were thaw on ice. 10 ng SC100 DNA were added to the bacteria cells, incubated for 20 minutes on ice, 2 minutes at 37 $^{\circ}$ C and again on ice for 2 minutes. Subsequently 400 μ l of Luria-Bertani broth medium (LB) (0.5% (w/v) NaCl, 1% (w/v) Trypton, 0.5% (w/v) Yeast-Extract, 2M Tris/HCl pH 7.5) were added and incubated for 1 hour at 37 $^{\circ}$ C. After 1 hour of incubation the cells were plated onto the LB-Agar plates containing 100 μ g/ml Ampicilin and incubated overnight at 37 $^{\circ}$ C. A single bacteria colony was selected for inoculation of 200 ml of LB-medium culture containing 100 μ g/ml Ampicilin. After an incubation of 18 hours at 37 $^{\circ}$ C at 180 rpm the bacterial culture was harvested by centrifugation at 4000 x g for 10 minutes at 4 $^{\circ}$ C (Allegra 25R-High-Performing-Centrifuge, TA-10-250 fixed angle rotor; Beckman). The DNA was purified using QIAGEN-EndoFree Plasmid Maxi Kit according to the manufacture's protocol. Extracted DNA was examined on 1% agarose gel after control digestion with AgeI and KpnI restriction enzymes (New England Biolabs) in order to confirm the propagation of the desired construct.

2.4. Flies

The flies were raised on standard fly food at 18 $^{\circ}$ C or 25 $^{\circ}$ C with 60-70% relative humidity.

2.4.1 *Drosophila* transgenic lines and mutants utilized in this work

Oregon R strain was utilized as wild-type control.

GMR-APP-GAL4, UAS-GRIM and GMR-GAL4, UAS-G/RPR transgenic flies were kindly provided by Dr. Ming Guo (Departement of Neurology, Brain Research Institute, The David Geffen School of Medicine, University of

California). GMR-APP-GAL4, UAS-GRIM transgenic system contains a type I chimeric protein containing human APP770 C-terminal fragment (APP770-Ct; initiating from the residue M671) that is appended to GAL4 on its C-terminal end (Figure 2.2.). APP770-Ct-GAL4 is specifically expressed in the eye under the control of the eye-specific GMR promoter. The reporter flies carry additionally to APP-GAL4, a γ -secretase reporter output construct, UAS-GRIM, that consists of a GAL4-responsive transcriptional cassette driving the expression of the *Drosophila* cell death activator GRIM. In GMR-GAL4, UAS-G/RPR flies GAL4 is expressed specifically in the eye under eye-specific GMR promoter but this transgenic strain do not contain APP770-Ct fragment. The expression of GRIM cell lethal gene is as above triggered through the activation of UAS by GAL4.

APP-GAL4-VP16, UAS-cd8-GFP transgenic strain was established in the laboratory of Dr. Dotti, by recombining the APP-GAL4-VP16 fusion flies (generous gift from Dr. Gary Struhl, Department of Genetics and Development, Howard Hughes Medical Institute, Columbia University) with UAS-cd8-GFP transgenic flies. This transgenic system contains APP695-C-terminal fragment (APP695-Ct) that initiates immediately downstream of β -cleavage site (residue M596). APP695-Ct is fused to the transcription factor GAL4-VP16 (GV) just downstream of the transmembrane domain (Figure 2.2.). After its intramembrane proteolysis APP C-terminal end the nucleus together with GV (APP-C-t-GV) and induces the expression of a nuclear UAS-cd8-GFP reporter gene.

APP770 and APP 695 isoforms do not differ in their C-terminal domains so that chimeric proteins in both transgenic strains GMR-APP-GAL4, UAS-GRIM and APP-GAL4-VP16, UAS-cd8-GFP have identical amino acid sequence (see figures 2.2. and 1.1. B).

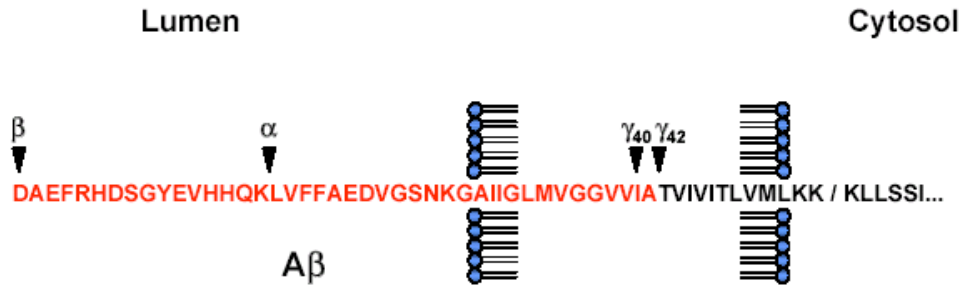


Figure 2.2. Amino acid sequence of the extracellular and transmembrane domain of chimeric protein form APP reporter flies. GMR-APP-GAL4, UAS-GRIM flies contain the APP770 C-terminal fragment; C-terminus of APP695 is contained in APP-GAL4-VP16 (GV), UAS-cd8-GFP transgenic flies. Both APP770 and APP695 C-terminal fragments are identical and encompass the extracellular domain that initiate directly after β -secretase cleavage site and the transmembrane domain. GAL4-VP16 transcription factor that is inserted just downstream of APP-Ct transmembrane domain is linked to the stop transfer signal through the last amino acids of APP-Ct (KK). Arrows indicate putative secretase cleavage sites of α -, β - and γ -secretase. Amino acids inside the A β domain is labelled in red.

Eas^{PC80f} mutant flies were a kind gift of Dr. Mark Tanouye (Department of Molecular and Cell Biology Division of Neurobiology, Department of Environmental Science, Policy, and Management, University of California). Ethanolamine kinase gene, termed *easily shocked* (*eas*) is localized on the X chromosome. The mutation in *eas^{PC80}* allele relies on a 2bp deletion at nucleotide position 1004-1005, which causes frame shift in the open reading frame leading to a stop codon in-frame at nucleotide position 1078. The peptide has only a portion of the conserved sequence and lacks completely the highly conserved kinase domain. *Eas^{PC80}* mutant gene is marked by a recessive expressed marker termed forked (*f*) that is localized on the X chromosome. *Forked* that causes

short bristles with split or bent ends was utilized to identify homozygous eas^{PC80} mutants.

2.4.2. Analysis of adult eye phenotype

Progeny transgenic for GMR-APP-GAL4, UAS-GRIM and homozygous for eas^{PC80f} mutation were obtained by crossing homozygous female eas^{PC80f} mutants with the male transgenic GMR-APP-GAL4, UAS-GRIM flies. Homozygous eas^{PC80f} progeny with following phenotype: **eas^{PC80f}/Y ; GMR-C99-GAL4, UAS-GRIM/+** and **eas^{PC80f}/eas^{PC80f} ; GMR-C99-GAL4, UAS-GRIM/+** were selected against *forked* marker and together with the control flies assayed for the adult eye roughness and the eye size. The eye size and the size of roughened eye surface are indicators for the level of retinal cell death that directly correlates with the expression of the cell lethal GRIM gene. Adult eye roughness was calculated as the percentage of the total eye surface. The eye size of adult progeny was measured in comparison to the wild-type flies and the transgenic flies without eas^{PC80} mutation. About 150 eyes/genotype were analysed by scoring images obtained by high-resolution light microscopy (Stereo microscope SZX9; Olympus). All measurements were performed in one-day-old adult flies. Progeny homozygous for eas^{PC80f} mutation, obtained by crossing male control transgenic flies GMR-GAL4, UAS-G/RPR with the female eas^{PC80f} mutants have a genotype as follows: **eas^{PC80f}/eas^{PC80f} ; GMR-GAL4, UAS-G/RPR/+**. These flies were selected against the marker *forked* and analysed for retinal cell death as described above.

2.4.3. Analysis of GFP expression

Homozygous eas^{PC80f} mutants transgenic for APP-GAL4-VP16, UAS-cd8-GFP (phenotype: **eas^{PC80f}/Y ; APP-GAL4-VP16, UAS-cd8-GFP/+**) were obtained by crossing the female eas^{PC80} homozygous with the male APP-GAL4-VP16, UAS-cd8-GFP transgenic flies. GFP expression in the progeny and control flies was

analysed by measuring the fluorescence intensity using Image J software or by Western blotting. In order to induce GFP expression one-day-old adult flies were heat shocked for 1 hour at 37°C, placed at 25°C to allow GFP expression and subsequently analysed by fluorescence intensity and Western blot assay. High-resolution light microscopy images were utilized for the analysis of fluorescence activity. For the Western blot analysis of the GFP protein expression 10 fly bodies were homogenized in lysis buffer containing 10 mM Tris/HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10% glycerol, 50 mM NaF, 1 mM NaVOF, 5 mM DTT, 4 M Urea, and protease inhibitors (CLAP), resolved by 15% Tris-glycine-acrylamide SDS-PAGE and analyzed as described under 2.2.3. using Anti-GFP-peptide antibody (BD Biosciences).

2.4.4. Membrane lipid analysis in flies

For the membrane lipid analysis 10 fly bodies (GFP reporter flies) or 20 fly heads (GRIM reported flies) were homogenized in lysis buffer (10 mM Tris/HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10% glycerol, 50 mM NaF, 1 mM NaVOF, 5 mM DTT, 4 M Urea, and protease inhibitors (CLAP)). After homogenization, the fly debris were centrifuged for 10 minutes by 18, 000 x *g* at 4°C, followed by the protein measurement in supernatant and cell membrane centrifugation by 100, 000 x *g* at 4°C for 1 hour, using the same amount of total protein as starting material for all samples. Subsequently the lipids were extracted and analysed as described under 2.2.6.

3. Results

3.1. Role of phosphatidylethanolamine (PE) in APP proteolytic processing in HEK 293 cells

3.1.1. Phosphatidylethanolamine distribution in the cell membranes of HEK 293 cells

In mammalian cells phosphatidylethanolamine (PE) represents one of the major membrane phospholipids. In addition to roles in normal cellular function, the possible involvement of PE in pathological situations, more precisely in AD, arose as consequence of the observation of altered PE levels in the brains of Alzheimer's patients (Wells et al., 1995, Prasad et al., 1997). While this is *per se* not an enough argument to strongly propose that PE are involved in the occurrence of AD (different from the changes being a consequence of it), this postulate can be also supported by the fact that, as mentioned in the chapter 1.2.2., PE is involved in membrane occurring enzymatic activities. This acquires further relevance if one accepts the view that one of the causes of AD is an increased amyloidogenic cleavage of APP, a membrane occurring event. As already described in the introduction, one of the by-products of APP cleavage is the neurotoxic A β peptide, whose accumulation in the brain mesenchyme is thought to be the cause of this disease. Therefore, we propose that changes in the PE content, can induce abnormal APP cleavage, leading to a perturbed production of A β . In further support of this view, APP was shown to colocalize with phospholipid rich membrane domains (Ehehalt et al., 2003, Abad-Rodriguez et al., 2004).

To gain insight into the above proposal I started by determining the distribution of PE in the membrane, measuring its levels in raft and non-raft domains. In order to determine PE distribution we utilized HEK 293 cells, the membranes of which were subjected to density gradient centrifugation. Gradient centrifugation allows the separation of cholesterol/sphingolipid rich phase from the phospholipid rich phase that constitutes the rest of the membrane.

The most widely used criterion for the separation of cholesterol/sphingolipid rich domains or rafts from non-rafts, is their insolubility in the nonionic detergent Triton X-100 at 4°C. After membrane solubilization in the nonionic detergent, raft associated lipids and proteins remain insoluble and can be floated to low density by OptiPrep gradient centrifugation.

To analyse the distribution of PE in the membranes of HEK 293 cells, cell membrane pellets were isolated by 100,000 x g centrifugation and subsequently solubilized at 4°C in a 1% Triton X-100 containing buffer. For the separation of detergent resistant or raft lipid microdomains (DRM) from the detergent soluble, non-raft microdomains, cell membrane pellets were loaded on an OptiPrep gradient and centrifuged at 120,000 x g for 18 hours at 4°C. To be certain that I had succeeded in the raft and non-raft isolation, the proteins from each gradient fraction were concentrated, re-suspended in sample buffer and subjected to 12% SDS-PAGE following by Western blotting for the detection of the raft associated protein, Flotilin-1. The interphase between 5% and 30% was identified as raft fraction, since the raft marker Flotilin-1 was significantly enriched in this gradient phase (Figure 3.1.1.). Further proof that rafts do become enriched in the 5% and 30% fractions of this type of gradient, comes from the observation that this is the fraction with most cholesterol (Figure 3.1.2).

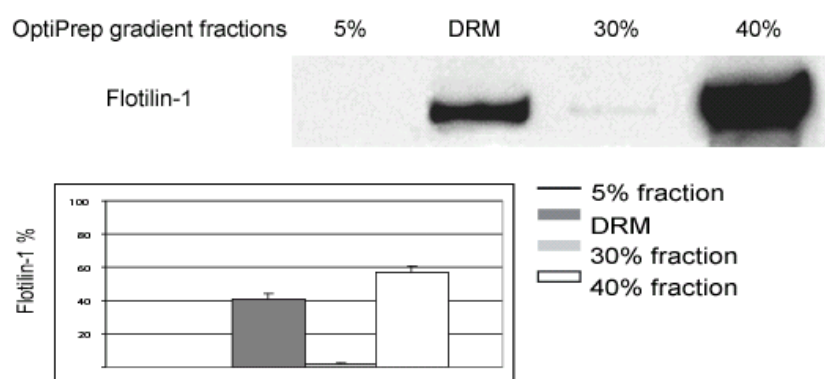


Figure 3.1.1. Flotilin-1 associates specifically with detergent insoluble membrane microdomains (DRM) in HEK 293 cells. Cellular membranes solubilized with 1% Triton X-100, were collected by flotation through the OptiPrep gradient. Subsequently,

the membranes of each gradient fraction were isolated by 100,000 x *g* centrifugation and subjected to 12% SDS-PAGE. Western blot analysis revealed an association of 41% of total Flotilin-1 with insoluble membrane fraction (DRM). As shown in the graph, 57% and 2% of total Flotilin-1 are detected in 40% and 30% fraction, respectively. The 5% fraction is not represented in the graph, since no Flotilin-1 was detected in this fraction.

After confirming the efficacy of the method for raft and non-raft fractions purification, PE distribution in these domains was analyzed by TLC. As before, cell membrane pellets were isolated by 100,000 x *g* centrifugation, followed by gradient centrifugation and membrane lipids extraction procedures (see Materials and Methods).

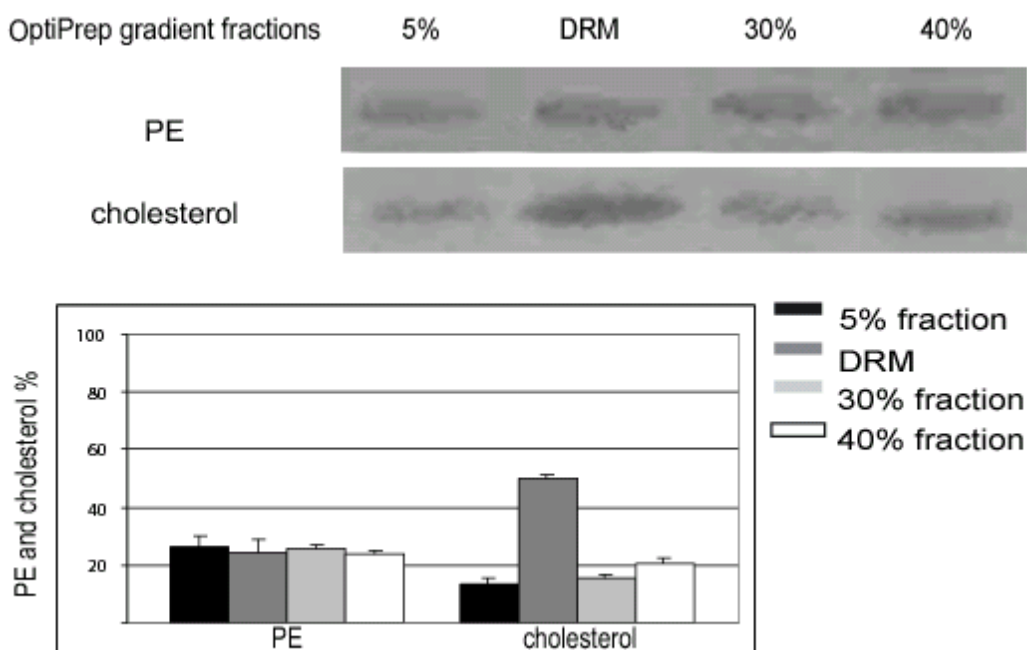


Figure 3.1.2. PE is equally distributed in the membranes of HEK 293 cells. After separation of membrane microdomains by OptiPrep gradient, membrane lipid extracts of DRM and non-DRM fractions were analysed by TLC. Lipid analysis showed that PE is equally distributed along the gradient, with about 25% of its total amount in each fraction. 75% of membrane PE is localized in non-raft domains, which encompass 5%, 30% and 40% fractions. DRM contain only 25% of PE. In contrast, the portion of total cholesterol was approximately 50% in the interphase between 5% and 30% fractions corresponding

to detergent insoluble microdomains (DRM). 5%, 30% and 40% gradient fractions contain 14%, 16% and 20% of total membrane cholesterol, respectively. The percentage of total PE and cholesterol in respective gradient fraction are depicted in the graph.

In the membranes of HEK 293 cells PE is equally distributed in raft and non-raft lipid microdomains, while cholesterol is, consistent to the Flotilin-1 localization, clearly enriched in the interphase between 5% and 30% gradient fractions (DRM) (Figure. 3.1.2.). PE ratio in non-rafts averages 0.75 versus only 0.25 in rafts, while the half (0.5) of total membrane cholesterol enriches in the raft fraction.

3.1.2. Localization of Amyloid Precursor Protein (APP) in the membranes of HEK 293 hAPP cells

Several studies have clearly indicated that membrane lipids are involved in pathogenesis of AD (Kuo et al. 1998, Jick et al. 2000, Wolozin et al.2000, Kivipelto et al.2001, Austen et al., 2002, Simons et al., 2002). In particular, cholesterol and cholesterol rich membrane microdomains seem to play an important role in APP proteolytic processing and generation of neurotoxic A β peptide (Simons et al., 1998, Simons et al., 2001, Fassbender et al., 2001, Eehalt et al., 2003, Abad-Rodriguez et al., 2004).

However, it was unequivocally shown, in both neuronal and non neuronal cells, that APP is, at endogenous levels, mostly, if not exclusively, present in the phospholipid rich/cholesterol poor membrane microdomains (Abad-Rodriguez et al., 2004), suggesting, without excluding the involvement of rafts, that non-raft domains may participate in the regulation of APP cleavage. Yet, before directly addressing this issue, I tested whether or not APP partitioning was, as in neuronal cells, restricted to non-raft domains in the HEK cells where I identified PE partitioning.

To determine APP membrane partitioning/localization, HEK 293 cells stably transfected with human APP were solubilized, and cell membrane pellets were isolated by centrifugation of cell extracts at 100,000 x *g* for 1 hour at 4⁰C. The

separation of raft from non-raft domains was performed as described, through density centrifugation in OptiPrep gradient. Then, the cell membrane pellets from each gradient fraction were isolated by 100,000 x g centrifugation, subjected to 10% SDS-PAGE and blotted with an APP C-terminal specific antibody (ANTI-APP, C-terminal, Sigma), to detect APP and identify its distribution along the gradient. Western blot analysis showed that in HEK 293 hAPP cells, APP is, consistently what described in neuronal cells, largely restricted to the non-raft, phospholipid rich domains (Figure 3.1.3.). 23% and 77% of total APP are present in the 30% and 40% fraction, respectively. Furthermore, APP was not detectable neither in DRM, the raft associated Flotilin-1 and cholesterol/sphingolipids enriched fraction, nor in the 5% fraction

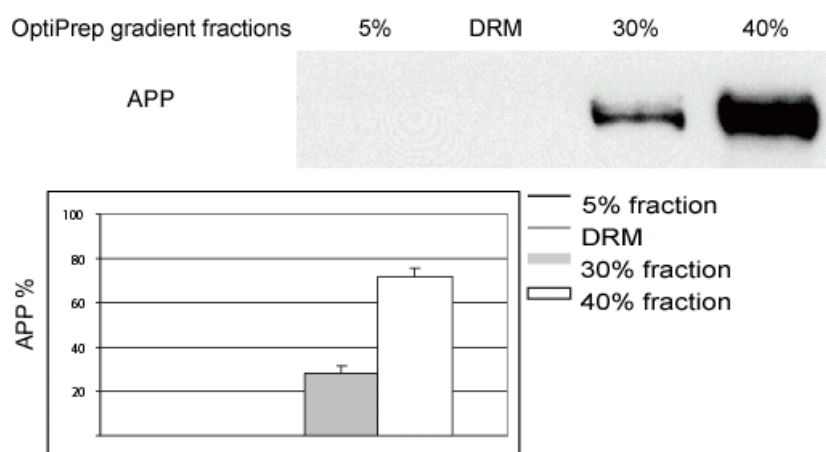


Figure 3.1.3. In the membranes of HEK 293 cells APP accumulates in the heavy, phospholipid rich fraction. After separation of DRM from non-DRM fractions by OptiPrep gradient, cell membranes of each fraction were isolated and subjected to 10% SDS-PAGE, to determine the distribution of APP along the gradient. Staining with ANTI-APP, C-terminal antibody reveals an accumulation of APP in the phospholipid rich fractions. Quantification of APP in the respective gradient fractions is represented in the graph. 23% and 77% of total APP is present in the 30% and 40% fraction, respectively. No APP was detected in the cholesterol rich domains (DRM) and the light 5% fraction.

These last results pave the way to undertake the functional study of the role of phospholipids-rich membrane domains in APP proteolytic processing.

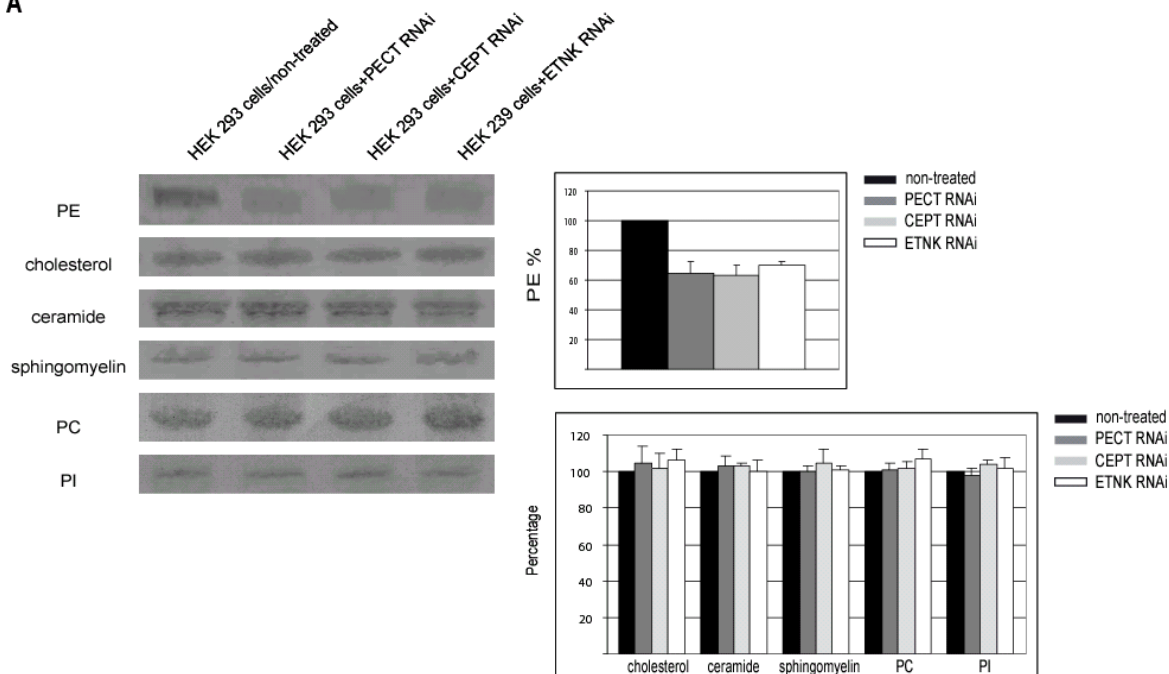
3.1.3. RNAi against PE synthesis enzymes in HEK 293 cells efficiently affect plasma membrane PE levels

To study the role of PE on APP proteolytic processing its membrane levels were modified by treatment with RNAi. The effect of altered PE levels on APP was studied by assaying the APP processing by α -, β - and γ -secretase. Since APP cleavage by γ -secretase is the crucial step in the generation of A β peptide, the interest was mainly focused on PE role in the regulation of this particular enzyme. In addition to γ -activity analysis, PE dependence of α - and β -secretase activity was studied, as amyloidogenesis appears to be regulated by the interaction of all three enzymes (see chapter 1.2.1.).

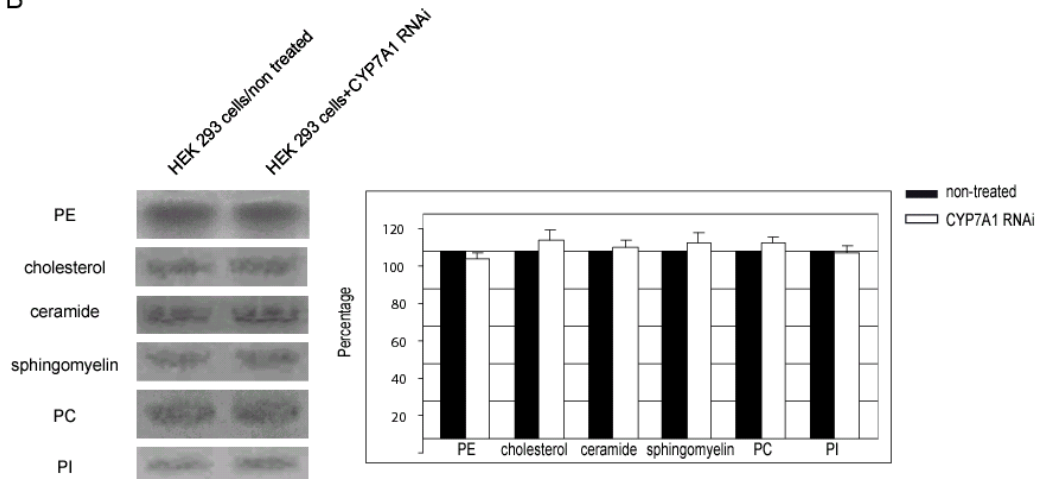
For the modification of membrane PE levels, following enzymes involved in the PE synthesis pathway were blocked by RNAi: phosphoethanolamine cytidyltransferase (PECT), CDP-ethanolamine phosphotransferase (CEPT) and ethanolamine kinase (ETNK). Cells were transfected with RNAi using Lipofectamine 2000 reagent according to the manufacturer's protocol and treated for 48 hours. RNAi efficacy was tested after 48 hours by measuring membrane PE levels in RNAi treated versus non-treated (control) cells (Figure 3.1.4. A). As a control, cells were treated with RNAi directed against an irrelevant messenger RNA (rat CYP7A1) (Figure 3.1.4. B). Cell membrane pellets isolation, followed by lipid extraction, lipid separation by TLC and lipid measurement, revealed that membrane PE level was approximately 40% lower in PECT, CEPT and ETNK RNAi treated cells compared to the non-treated cells, whereas the membrane content of cholesterol, ceramide, sphingomyelin, PC and PI did not significantly differ in treated and non-treated cells (Figure 3.1.4. A). The treatment with the control CYP7A1 RNAi had no effect on membrane lipid content (Figure 3.1.4. B). To test whether RNAi treatment and PE depletion have a deleterious affect on the cells, cell viability was assayed by measuring apoptosis and necrosis in low PE cells versus control cells with wild type PE levels (Figure 3.1.4. C). As both apoptosis and necrosis were not significantly different in PE depleted and control

cells, I could rule out that PE depletion may have an unspecific effect on APP proteolytic processing through inducing cell death.

A



B



C

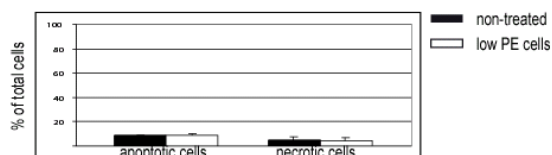


Figure 3.1.4. HEK 293 cells treated with RNAi, directed against enzymes involved in PE synthesis pathway show a decrease of membrane PE levels. HEK 293 cells were treated with RNAi against PECT, CEPT and ETNK and the control RNAi, directed against rat CYP7A1. After 48 hours of RNAi treatment cell membranes were isolated, using the same amount of total protein as starting material. Membrane lipids were extracted and analyzed by TLC. Lipid quantification is shown in the graph. **(A)** HEK cells treated with the RNAi directed against PECT, CEPT and ETNK show approximately 40% lower membrane PE content than non-treated cells. Membrane levels of cholesterol, ceramide, sphingomyelin, PC and PI do not significantly differ in non-treated and RNAi treated cells. **(B)** HEK 293 cells, treated with the control, rat CYP7A1 RNAi, under the same experimental conditions like the cells described in A, do not show any significant changes in the membrane lipid composition in comparison to the non-treated cells. **(C)** Apoptosis levels, measured by TUNNEL assay have similar values, 9% of total cells, in non-treated and PE depleted cells. Also necrosis, determined by Trypan blue staining, was not enhanced by PE depletion. The amount of necrotic cells in control (non-treated) and low PE cells was about 3% of total cells.

3.1.4. RNAi against PE synthesis enzymes in HEK 293 cells alter APP cleavage efficacy

To determine whether PE depletion affects proteolytic processing of APP, low PE HEK 293-wtAPP695 cells were analysed for the changes in APP cleavage by α - β -, and γ -secretase. Cleavage of APP by α - or alternatively by β -secretase leads to generation and extracellular release of soluble APP peptides, sAPP α and sAPP β , respectively, and the retention of corresponding membrane-anchored C-terminal fragment, α CTF (C83) and β CTF (C99). Membrane-anchored C-terminal fragments α CTF and β CTF, are both substrates for γ -secretase that cleaves C83 and C99 within the membrane and derives nonamyloidogenic p3 and amyloidogenic A β , respectively. Additionally to p3 and A β , γ -secretase cleavage releases AICD in the intracellular space.

To analyse the effect of PE depletion on α - and β -secretase cleavage of APP, HEK 293 cells stably expressing wild-type APP695 were treated with RNAi

directed against the enzymes: PECT, CEPT and ETNK, as described above. Crude membrane pellets, from non-treated (control) and RNAi treated cells, were resolved by the urea version of the Bicine/bis-Tris/Tris/sulfate SDS-PAGE (Wiltfang et al., 1991). Western blot analysis was performed with ANTI-APP, C-terminal antibody to visualize full length APP (fl APP) and the membrane-anchored α CTF. β CTF fragment was detected by Anti-Human β Amyloid [1-17] antibody. In RNAi treated cells, with approximately 40% less membrane PE, levels of APP-C-terminal fragment generated by β -secretase processing of APP, revealed a 40-50% decrease compared to the cells with wild-type PE levels (Figure 3.1.5. A). α CTF levels in low membrane PE cells were increased an average of 50% over the control. In addition to α CTF analysis, α -secretase activity was assayed by changes in the levels of secreted sAPP α . Correspondingly to the increased α CTF levels, sAPP α content in the medium of low PE cells was about 50% higher, with respect to the control cells (Figure 3.1.5. A). To rule out, that the changes in the generation of α CTF and β CTF are due to the alterations in APP expression the levels fl APP were determined in low PE cells versus control. Quantification of fl APP normalized to the total amount of α Tubulin, revealed that, PE depletion in the membranes of HEK 293 cells did not cause alterations in APP content. Control RNAi directed against rat CYP7A1 had an effect neither on α CTF and β CTF nor on fl APP levels (Figure 3.1.5. B), which excludes an unspecific effect of RNAi on APP proteolytic processing. Taken together the results above indicate a specific effect of PE on α - and β -secretase activity on APP.

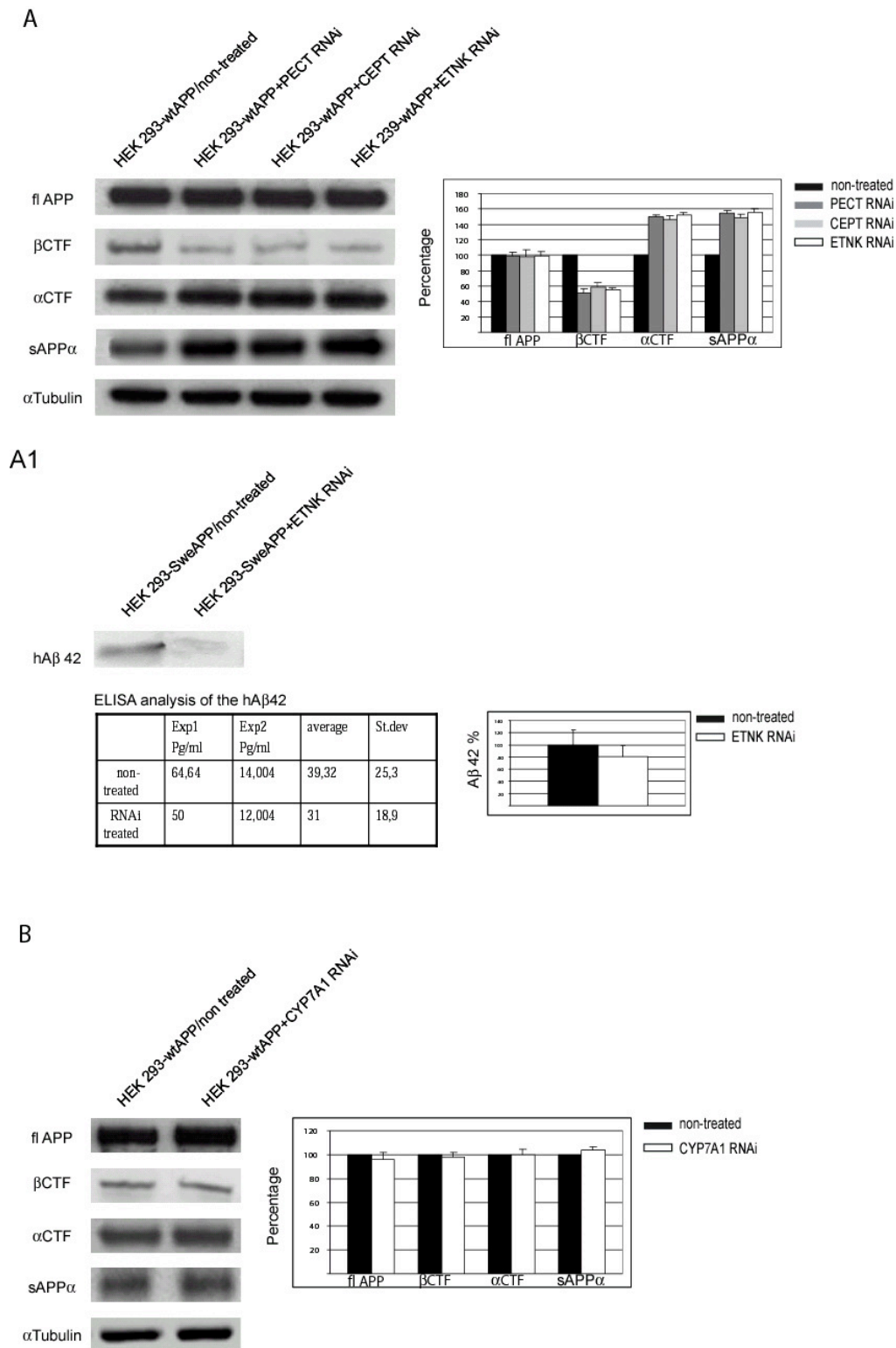


Figure 3.1.5. HEK 293 cells with low membrane PE show changes in APP processing by α -, β - and γ -secretase. (A) Activity of α -, β - and γ -secretase in HEK

293-wild-type APP695 cells was analysed after depletion of approximately 40% of membrane PE by RNAi directed against PECT, CEPT and ETNK enzymes. For determination of fl APP, β and α CTF's crude membrane pellets isolated from non-treated (control) and RNAi treated cells were subjected to the urea version of the Bicine/bis-Tris/Tris/sulfate SDS-PAGE. Tubulin served as the loading control. The amount of fl APP, determined by ANTI-APP C-terminal antibody does not significantly differ in control and low PE cells. Membrane levels of β CTF fragment, determined by Anti-Human β Amyloid [1-17] antibody, were 40- 50% lower in PE depleted cells compared to the control. Analysis of the membrane-anchored α CTF, visualized by ANTI-APP C-terminal antibody revealed that the amount of α CTF in low PE cells was approximately 50% increased with respect to the control cells. α CTF and β CTF membrane levels, determined by densitometry were normalized to the amount of total APP. Consistent to the elevated levels of α CTF, the amount of sAPP α in the medium was about 50% higher than in the medium from the control cells. Immunoprecipitations from extracellular medium were submitted to 10% Laemmli-PAGE and sAPP α visualized by A β N-terminus specific antibody 1E8 (Nanotools). **(A1)** In HEK 293-SwedishAPP695 A β 42 generation was analysed in ETNK RNAi treated cells versus control, by ELISA and Western blotting using WO2 antibody. Compared to the non-treated cells, RNAi treated cells show a decrease in A β generation of approximately 18%. Quantification of A β 42 peptide, depicted in the graph, revealed similar results in both assays. **(B)** Control RNAi against rat CYP7A1 did not cause any alterations in full length APP levels and its proteolytic processing by α - and β -secretase. The analyses of fl APP, β and α CTF's and sAPP α were performed as described under A. Quantifications are shown in the respective graph.

After it was shown that membrane PE depletion is related to the changes in the generation of α - and β -secretase products (Figure 3.1.5.), γ -secretase activity was analysed, measuring changes in the A β production. For the analysis of A β , cell culture medium from the PECT, CEPT and ETNK RNAi treated and control cells was collected and A β peptide recovered by immunoprecipitation with Amyloid beta A4, amino-terminal-selective antibody 1E8 (Nanotools). Subsequently immunoprecipitates were analysed on the urea version of the Bicine/bis-Tris/Tris/sulfate SDS-PAGE, using the A β 1E8 antibody.

The antibody detection of A β released into the extracellular medium from the HEK 293-wild-type APP695 cells showed that the amount of peptide was not sufficient to perform a proper quantification. In order to overcome this problem I switched the A β analysis to the HEK cells, which overexpress human APP695 with the *Swedish* mutation (SweAPP695). This mutation in the *APP* gene elevates the amount of generated A β that allows a valid quantification of the peptide. PE levels in the membranes of HEK 293-SweAPP695 cells were depleted by RNAi directed against the ETNK enzyme. Afterwards A β generation was analysed by Western blotting and ELISA assay in low PE cells versus non-treated (control) cells, with wild-type membrane PE levels. For Western blot detection of A β peptide extracellular medium (the amount was standardized by the protein amount) was re-suspended in sample buffer and resolved by the 16.5% SDS-PAGE. A β peptide was detected by using the WO2 antibody that is raised against the residues 5-8 of A β region (Ida et al., 1996). The antibody detection revealed that in HEK 293-SweAPP695 cells with depleted membrane PE, the levels of the neurotoxic A β 42 species show a decrease of about 20% in comparison to the control cells. An additional quantification of human A β 42 in the medium was performed using the highly sensitive kit “hAmyloid β 42 ELISA (HS)” (the GENETICS company) following the manufacturers instruction. The results obtained by ELISA confirmed those from Western blot assay, revealing an A β 42 decrease of approximately 20% after membrane PE depletion (Figure 3.1.5. A1).

Since the data in HEK 293 cells clearly show a correlation of membrane PE levels and APP processing, experimental investigations were switched to a more sophisticated *Drosophila melanogaster in vivo* system, in order to prove the results obtained in mammalian *in vitro* system.

3.2. Role of phosphatidylethanolamine (PE) in APP proteolytic processing in *Drosophila melanogaster*

Drosophila melanogaster is a widely used model system to study neurodegenerative diseases, including Alzheimer's disease. The strength of *Drosophila* as an experimental organism relies on its powerful genetic tools (e.g. large amount of transgenic lines and mutants), intensive genetic analysis of its genome, short generation time and nevertheless the fact that *Drosophila* and humans share many structurally and functionally related gene families, despite their phylogenetic distance. One of these, in *Drosophila* and mammals, highly conserved metabolic pathways is the mechanism of γ -secretase activity (Fossgreen et al., 1998, Takasugi et al., 2002). γ -secretase has been shown to play the main role in the generation of A β peptide, which forms senile plaques, hallmark of AD pathology. Furthermore α -secretase-like activity has been reported in *Drosophila* (Fossgreen et al., 1998, Loewer et al., 2004).

It is noteworthy that amyloid precursor protein-like protein APPL, a *Drosophila* homologue of hAPP does not exhibit sequence similarities within the β -amyloid region of APP (Selkoe, 1996), and γ -cleavage of APPL has not been documented (Rosen et al., 1990). But it has been shown that γ -secretase in *Drosophila* cleaves overexpressed hAPP and hAPP-C-terminal fragment in the similar manner like in mammals, under generation of A β peptide and APP intracellular domain (AICD) (Fossgreen et al., 1998, Takasugi et al., 2002, Loewer et al., 2004).

Localization of APP and APP cleaving machinery on the membrane, suggests a role of membrane lipids in the regulation of APP processing. As described in the introduction, APP is processed by three proteases termed α -, β - and γ -secretase. A β peptide is produced by the sequential cleavage of APP by β - and γ -secretase (Annaert and De Strooper, 2002). This APP processing pathway is termed amyloidogenic pathway. The production of an intact A β peptide is

prevented by APP processing through the non-amyloidogenic pathway, in which α -secretase cleaves APP within A β domain.

In chapter 3.1. I showed that in mammalian HEK 293 cells, membrane levels of PE clearly correlate with processing of APP by α -, β - and γ -secretase. Here I will describe how the modulation of PE levels affect APP cleavage *in vivo*. For this I have utilized *Drosophila melanogaster*, which, as mentioned above has been shown to be valuable to study this phenomenon (Fossgreen et al., 1998, Lichtenthaler et al., 1999, Struhl and Adachi, 2000, Takasugi et al., 2002, Francis et al., 2002, Loewer et al., 2003, Guo et al., 2003, Doglio et al., 2006). Moreover, *Drosophila* has been as well useful to study PE modulation (Pavlidis et al., 1994, Dobrosotskaya et al., 2000, Nyako et al., 2001).

My *in vivo*, *Drosophila*-based validation work, started using embryonic Schneider-2 (S2) cells grown *in vitro*, transiently transfected with SC100, an APP-C-terminal-domain (C99) containing construct (SC100: M596-N695 of APP695, see Material and Methods, Figure 2.1.). For the true *in vivo* studies I utilized transgenic fly lines expressing the C-terminal fragment of hAPP (see Material and Methods, Figure 2.2.) and the eas^{PC8f} mutant, defective in the gene for ethanolamine kinase.

3.2.1. Role of phosphatidylethanolamine (PE) in APP proteolytic processing in *Drosophila* S2 cells

3.2.1.1. Phosphatidylethanolamine distribution in the cell membranes of *Drosophila* S2 cells

Like in mammalian cells, γ -secretase cleavage in *Drosophila* occurs on the membrane. Phospholipids represent, besides sterols and sphingolipids, main lipid species in *Drosophila* membranes. Barring the some differences, e.g. in the length of fatty acyl chains, phospholipids in mammals and *Drosophila* have a similar organization. PE, which is shown to correlate with the changes in APP

processing in HEK 293 cells (see above), comprises 55% of total phospholipids in *Drosophila* (Jones et al., 1992). Similar to the situation in mammalian cells, structural properties of membrane lipids in *Drosophila* cells are consistent with the ability to separate into sterol/sphingolipid rich, liquid ordered and phospholipid rich, liquid disordered phase (Rietveld et al., 1999). Ergosterol/sphingolipid rich domains in *Drosophila* cells have been shown to be insoluble in nonionic detergent at 4°C, other than phospholipid rich membrane domains, which are detergent soluble.

In order to study the role of PE in hC99 processing its membrane distribution in S2 cells was determined by density gradient centrifugation. S2 membranes were solubilized for 1 hour at 4°C in a 1% Triton X-100 containing buffer and subsequently loaded on OptiPrep gradient, to separate detergent resistant microdomains (DRM) from detergent soluble fractions (non-DRM). *Drosophila melanogaster* Flotilin (Flotilin^{Dm}), which is closely related to the raft associated, mammalian Flotillin-1 (Galbiati et al., 1998), was used to identify DRM or raft microdomains. For the identification of DRM, I performed a gradient phase separation by 120.000 x g at 4°C for 18 hours. Subsequently, membrane pellets from each fraction were extracted, re-suspended in sample buffer and loaded to 12% SDS-PAGE. Detection of Flotilin^{Dm} was performed by Western blotting using Flotillin-1 antibody. Flotilin^{Dm} was significantly enriched in the interphase between 5% and 30% OptiPrep gradient fractions (Figure 3.2.1.). This data, together with ergosterol enrichment in the 5%-30% fraction interphase (Figure 3.2.2.), indicates flotation density of DRM in this gradient fraction.

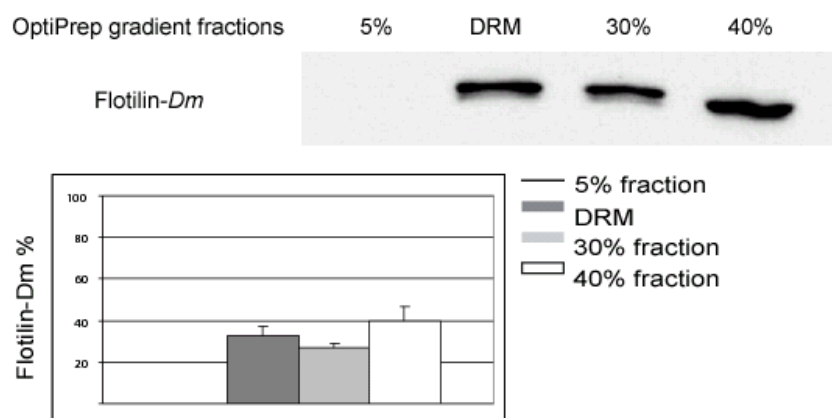


Figure 3.2.1. *Drosophila* Flotilin (Flotilin^{Dm}) is abundant in detergent insoluble membrane microdomains (DRM) of S2 cells. Distribution of Flotilin^{Dm} was analysed by density gradient centrifugation of S2 membranes, solubilized for 1 hour at 4°C in a 1% Triton X-100 containing buffer. OptiPrep gradient centrifugation was performed at 120,000 x g for 18 hours at 4°C. After separation of raft and non-raft microdomains, gradient fractions were collected. Cell membranes of each fraction were isolated by 100,000 x g, resuspended in sample buffer and subjected to 12% SDS-PAGE. As depicted in the graph, 33% of total Flotilin^{Dm} was present in the interphase between 5% and 30% gradient fraction. Measurement of Flotilin^{Dm} levels in other gradient fractions show that 27% and 40% of total Flotilin^{Dm} is localized in 30% and 40% fractions, respectively. No Flotilin^{Dm} was detected in the light 5% fraction.

To determine PE distribution in S2 membranes, cell membrane pellets, solubilized in 1% Triton X-100, were separated in OptiPrep gradient as described above. After density centrifugation, membranes from each gradient fraction were isolated by 100,000 x g, and membrane lipids extracted according to Bligh and Dyer (1959). Subsequent analysis of PE distribution by TLC shows that PE is equally distributed all over the gradient, while ergosterol enriches in the 5%-30% interphase, consistent to the Flotilin^{Dm} localization. The results concerning the membrane distribution of PE and ergosterol obtained in S2 cells were similar to those in mammalian cells. The ratio of total PE is 0.75 in non-rafts, while its portion in rafts averages only 0.25. Other than PE, ergosterol is abundant in raft fraction with 0.45 of its total membrane content.

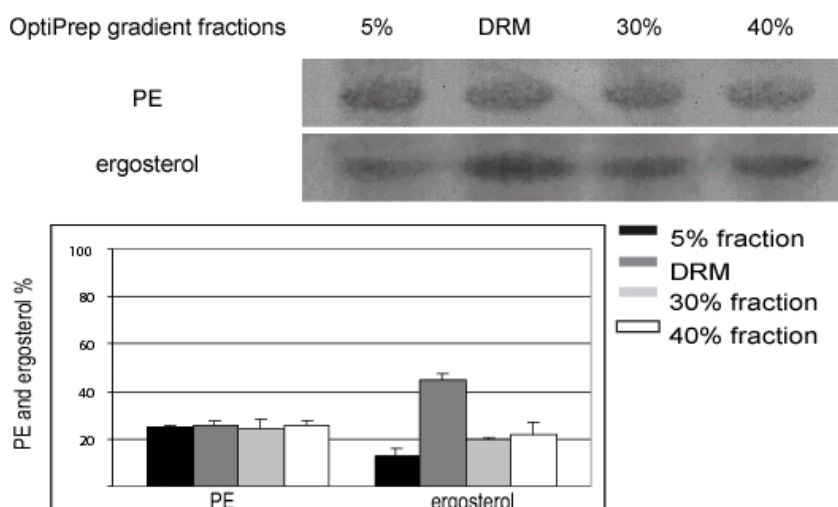


Figure 3.2.2. In *Drosophila* S2 cells 75% of total membrane PE is present in non-raft fraction. S2 cell membranes were solubilized with 1% Triton X-100 at 4°C and loaded on OptiPrep gradient to determine PE distribution. After separation of detergent soluble from detergent insoluble membranes by density centrifugation, membranes from each gradient fraction were isolated by 100,000 x g centrifugation and analysed by TLC. The ratios of total membrane PE in respective gradient fraction, which are depicted in the graph, show that all gradient fractions contain similar amount of approximately 25% of total membrane PE. Non-raft domains, encompassing the 5%, 30% and 40% fractions, contain 75% of total membrane PE. Contrariwise, ergosterol is with 45% of its total amount strongly enriched in the raft fraction, identified by Flotilin^{Dm} in the 5%-30% interphase. In 5%, 30 % and 40% fractions ergosterol was present with 13%, 20% and 22%, respectively.

3.2.1.2. Localization of hAPP (hC99) in the cell membranes of *Drosophila* S2 cells

To investigate, whether in S2 cells, membrane PE plays a role in APP processing, as shown in HEK 293 cells, the distribution/localization of hC99 in the membranes of S2 cells was analysed. S2 cells were transiently transfected with hAPP695 C-terminal-domain (hC99) containing construct (SC100, Maryama et al., 1994). The transfection was performed with Calcium Phosphate method, according to the manufacturer's protocol (Invitrogen). 72 hours after transfection, cell membranes were solubilized in 1% Triton X-100 and submitted to the OptiPrep gradient to determine hC99 distribution along the gradient. After 18 hours centrifugation by 120,000 x g, gradient fractions were collected, membrane extracts of each fraction isolated by 100,000 x g centrifugation and subjected to the urea version of the Bicine/bis-Tris/Tris/sulfate SDS-PAGE. The localization of hC99 was determined by immunostaining with ANTI-APP C-Terminal antibody. As depicted in the Figure 3.2.3., about 84% of total hC99 was detected in 40% gradient fractions. 30% fraction contains 16% of total hC99, whereas no hC99 was detected in 5% and DRM fraction. Like in mammalian cells, localisation of hAPP (hC99) in *Drosophila* S2 cells is mainly restricted to the phospholipid rich

fraction, unlike Flotilin and ergosterol distribution, which are significantly enriched in DRM fraction.

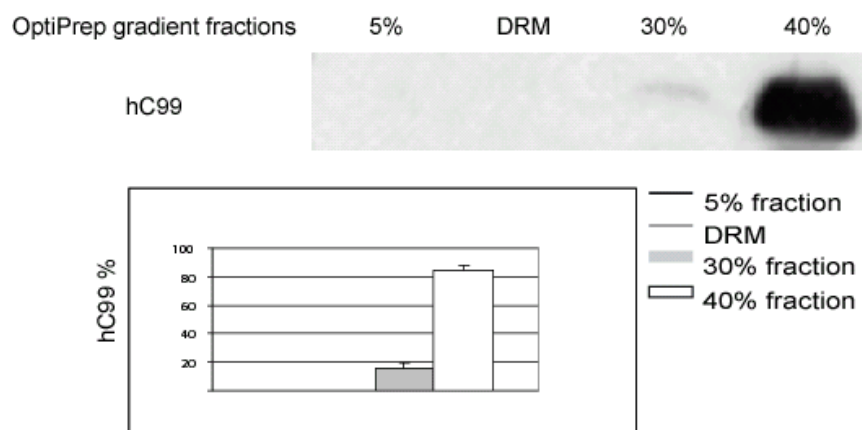


Figure 3.2.3. Overexpressed hC99 is mainly enriched in the non-raft microdomains of *Drosophila* S2 cells. Raft and non-raft microdomains of S2 cells overexpressing hC99, were isolated by OptiPrep gradient centrifugation. Subsequently cell membranes from each fraction were isolated, resuspended in sample buffer, resolved by the urea version of the Bicine/bis-Tris/Tris/sulfate SDS-PAGE and stained with ANTI-APP C-Terminal antibody, to analyse the localization of hC99. Quantification, shown in the graph, revealed that 84% of total hC99 is present in 40% fraction. Approximately 16% of hC99 was detected in 30% fraction, whereas no hC99 was found DRM and 5% gradient fractions.

Enrichment of hC99 within the phospholipid rich membrane core strongly suggests an important role of those lipids in C99 processing. Among all phospholipids PE appears most interesting regarding its role in cellular processes. Moreover PE constitutes 55% of total phospholipids in *Drosophila* membranes.

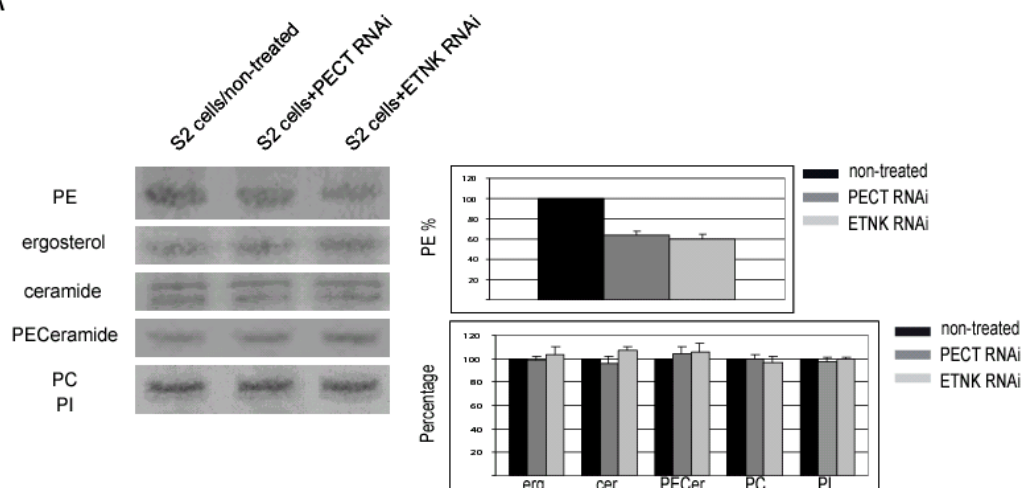
3.2.1.3. Altered membrane PE levels in *Drosophila* S2 cells correlate with changes in hC99 processing

The fact that, in mammalian and in *Drosophila* cells, hAPP (hC99) preferentially colocalizes with phospholipid rich membrane domains, strongly indicates the role of those lipids in the regulation of APP processing. In *Drosophila*, *in vitro* and *in vivo* systems, PS-dependant γ -secretase activity has been shown to be highly conserved and to cleave overexpressed hAPP (Fossgreen et al., 1998, Takasugi et al., 2002, Loewer et al., 2004). Besides γ -secretase cleavage, hAPP in *Drosophila* is processed by α -secretase-like activity, that generates a ~ 10-kDa polypeptide comigrating with C83 fragment of mammalian cells. (Fossgreen et al., 1998, Takasugi et al., 2002, Loewer et al., 2004). Processing of hAPP-Ct by *Drosophila* γ -secretase generates A β peptide and the APP intracellular domain (AICD).

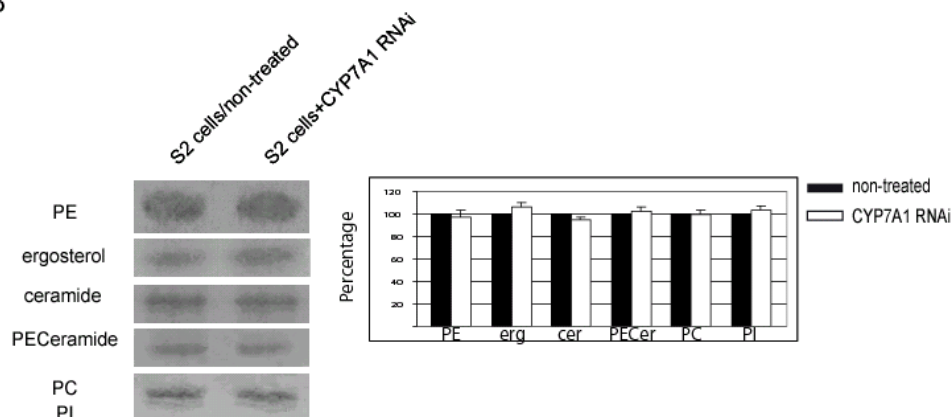
In order to determine the role of PE in APP processing by γ -secretase, A β levels were analyzed in low PE cells versus control cells with wild type membrane PE levels. In addition to A β generation I assayed the changes in hC99 and α -stub-like fragments, as they correlate with the levels of γ -secretase activity. To deplete membrane PE, S2 cells were treated with RNAi directed against enzymes involved in the CDP-Ethanolamine pathway: phosphoethanolamine cytidyltransferase (PECT) and ethanolamine kinase (ETNK). At first *Drosophila* S2 cells were transiently transfected with hC99. 24 hours after transfection cells were treated with RNAi for 48 hours. Such treatment abolishes the corresponding endogenous mRNA selectively through RNAi and leads to the loss of respective enzyme activity. After 48 hours of RNAi treatment, membrane pellets from treated and non-treated (control) cells were isolated by 100,000 x g and after lipid extraction analysed by TLC. Membrane lipid analysis revealed that membrane PE content in PECT and ETNK RNAi treated cells was about 40% lower with respect to non-treated cells (Figure 3.2.4. A). In contrast, membrane content of ergosterol, ceramide, PECeramide, phosphatidylcholine (PC) and phosphatidylinositol (PI) was not significantly altered compared to the non-treated

cells (Figure 3.2.4. A). To exclude an unspecific effect of RNAi on membrane PE content S2 cells, transiently transfected with hC99 were treated for 48 hours with a control RNAi, directed against rat CYP7A1 mRNA. Membrane isolation, lipid extraction and analysis were carried out under the same experimental conditions like above. As shown in the Figure 3.2.4. B, control RNAi did not cause any changes in the membrane lipid composition of S2 cells. Cell viability, tested by apoptosis and necrosis in low PE versus control cells, revealed that the levels of cell death in PE depleted and control cells did not significantly differ, i.e. PE depletion does not have a deleterious effect on S2 cells (Figure 3.2.4. C).

A



B



C

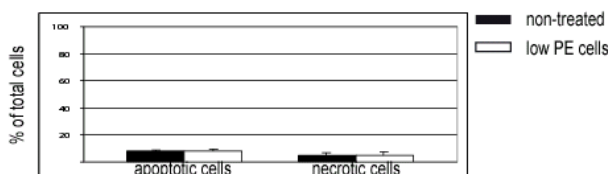


Figure 3.2.4. S2 cells show approximately 40% decreased membrane PE levels after treatment with RNAi directed against PECT and ETNK enzymes. (A) 48 hours after PECT and ETNK RNAi treatment, extracts from S2 cells overexpressing hC99 were centrifuged for 1 hour at $100,000 \times g$ in order to isolate cell membrane pellets. Membrane lipids extracted from crude membranes of RNAi treated and non-treated cells were analysed by TLC. Lipid quantification in RNAi treated cells versus non-treated (control) cells revealed that PECT and ETNK RNAi treatment induced a decrease of membrane PE of approximately 40% with respect to the non-treated cells. Ergosterol, ceramide, PECeramide, PC and PE do not show significantly different membrane levels in RNAi treated and non-treated cells. (B) S2 cells treated with control rat CYP7A1 RNAi, under same experimental conditions as described above, do not exhibit any changes in their membrane lipid levels in comparison to the non-treated cells. Quantifications of membrane lipid content in RNAi treated cells versus control are shown in the graph. (C) Levels of apoptosis and necrosis measured in low PE cells did not reveal significant differences to control cells. As shown in the graph the percentage of apoptotic cells was about 8% of total cells in PE depleted cells and the cells with wild-type PE content. Also the level of necrosis in both, low PE and control cells was about 5% of total cell amount.

After it was shown, that PECT and ETNK RNAi treatment specifically leads to PE depletion, without significant alterations in membrane content of other major lipids and without affecting cell viability, I addressed the question, whether PE depletion causes changes in hC99 proteolytic processing. For the analysis of membrane-anchored C99 and α -stub-like fragments, crude membrane pellets from non-treated and low membrane PE cells were isolated, subjected to the urea version of the Bicine/bis-Tris/Tris/sulfate SDS-PAGE and stained with ANTI-APP, C-Terminal antibody. Immunoblot analysis showed a decrease of approximately 65% in C99 and α -stub-like levels, in low PE cells with respect to the non-treated (control) cells (Figure 3.2.5., left panel). Both membrane-anchored fragments, C99 and α -stub-like are substrates for γ -secretase and the decrease in their membrane levels indicates that they are longer processed by γ -secretase than under control conditions. This observation indicates an elevated γ -secretase activity but as C99 and α -stub-like membrane levels are only indirect

indicators for γ -secretase activity, I analysed the amount of A β in order to directly assay the effect of PE depletion on γ -activity.

A β peptide was immunoprecipitated from the extracellular medium of the control and PE depleted cells, using Amyloid beta A4, amino-terminal-selective antibody 1E8 (Nanotools) and afterwards analysed on the urea version of the Bicine/bis-Tris/Tris/sulfate SDS-PAGE. As already indicated by C99 and α -stub-like analysis, A β generation changes dramatically after membrane PE depletion. The amount of total A β in low PE cells averages 80% over the control (Figure 3.2.5. B). PE depletion did not alter the rates of single A β species in relation to the total A β content. Figure 3.2.5. (right panel) depicts different A β species, generated depending on γ -secretase cleavage site. "A β X" represents an A β peptide species that is not defined yet (personal comment from Dr. Wiltfang).

Crude membrane pellets and the cell culture medium from CYP7A1 RNAi treated and non-treated cells were analysed for C99, α -stub-like fragment and A β as described above. Western blot analyses revealed that rat CYP7A1 RNAi did not induce any significant changes neither in C99 and α -stub-like nor in A β generation (Figure 3.2.5., see left and right panel).

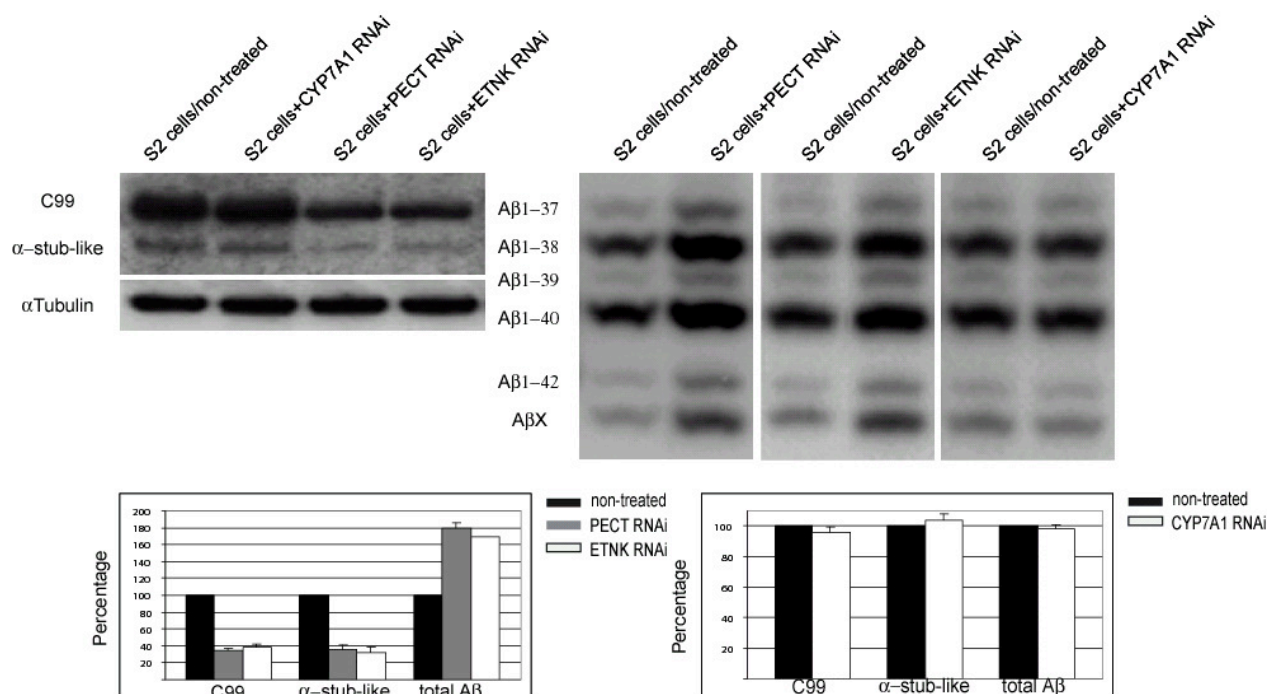


Figure 3.2.5. In S2 cells, membrane PE depletion by PECT and ETNK RNAi leads to alterations in hC99 processing by γ -secretase. (left panel) Analysis of crude membrane pellets, isolated from PECT and ETNK RNAi treated S2 cells show altered levels of C99 and α -stub-like fragments versus control cells. Both membrane-anchored fragments were analysed using urea version of the Bicine/bis-Tris/Tris/sulfate SDS-PAGE and ANTI-APP C-Terminal antibody. In comparison to the non-treated cells, membranes of PECT and ETNK RNAi treated cells show about 65% lower level of C99 and α -stub-like fragments. Densitometry measurements were normalized to the amount of α Tubulin. **(right panel)** Immunoprecipitations from extracellular medium were resolved by the urea version of the Bicine/bis-Tris/Tris/sulfate SDS-PAGE and visualized using Amyloid beta A4, amino-terminal-selective antibody 1E8. The densitometry analysis revealed that total A β content in PE depleted cells averages approximately 80% over the control. The ratios of single A β species (A β 1-37, A β 1-38, A β 1-39, A β 1-40, A β 1-42, A β X) do not significantly differ in control and low PE cells. **(left and right panel)** Control treatment, performed under the same experimental conditions as described above with rat CYP7A1 RNAi had a significant effect neither on levels of membrane-anchored APP fragments, nor on generation of A β peptide.

Increased generation of A β peptide as well as decreased C99 and α -stub-like membrane levels, described above, indicate an elevated activity of γ -secretase on APP caused by PE depletion. These data contradict the results obtained in mammalian system, where I had shown that reduction of membrane PE content leads to decreased APP proteolytic processing by γ - and β -secretase. One possibility for the discrepancy would be that, because of being embryonic, Schneider-2 cells represent PE-mediated modulation of APP at the embryonic, but not mature stage of life. Another possibility is that *Drosophila* cells have *in vitro* requirements not relevant in the more complex situation of the *in situ* scenario. Either possibility could be addressed by looking at the processing of hC99 in *Drosophila in vivo*. The results of this analysis are described next.

3.2.2. Role of phosphatidylethanolamine (PE) in APP proteolytic processing *Drosophila in vivo* system

As already mentioned above, hAPP processing in *Drosophila* system has been described in several studies (Fossgreen et al., 1998, Lichtenthaler et al., 1999, Struhl and Adachi, 2000, Loewer et al., 2003, Guo et al., 2003, Doglio et al., 2006). To study the role of PE in APP processing, I have utilized *Drosophila melanogaster* transgenic strains expressing hAPP-C-terminal (APP-Ct) fragment initiating downstream of the β -secretase cleavage site. These γ -reporter flies were crossed with eas^{PC80f} mutant, defective in the gene for ethanolamine kinase.

One of *Drosophila* transgenic lines used in this study is **GMR-APP-GAL4, UAS-GRIM** which acts as a reporter of γ -secretase activity in the fly eye (Guo et al., 2003). These reporter flies, allow the studying of endogenous levels of γ -secretase activity, using a nonessential neuronal tissue, adult fly eye. As γ -secretase substrate serves a chimeric type-I transmembrane protein, which contains a N-terminus, with a cleavable signal sequence followed by human APP-C-terminal fragment to the β -secretase cleaving site (see Material and Methods, Figure 2.2.). The protein is targeted to the secretory pathway by the N-terminal signal sequence. On its C-terminal end hAPP-Ct is fused to the yeast transcription factor GAL4. APP-Ct-GAL4 is specifically expressed in the eye under control of eye specific GMR promoter. The reporter flies carry additionally to hAPP-Ct-GAL4, a γ -secretase reporter output construct, UAS-GRIM, that consists of a GAL4-responsive transcriptional cassette driving the expression of the *Drosophila* cell death activator GRIM (Chen et al., 1996). After γ -secretase cleavage, APP-C-terminal fragment downstream of the γ -cleavage site moves together with GAL4-VP16 to the nucleus, and triggers the transcription of cell lethal gene GRIM, through UAS activation (Figure 3.2.8. G) Activation of Grim transcription by γ -secretase processing of hAPP-Ct, promotes cell death in the fly eye, so that the size of the eye and the roughened surface correlate with the level of endogenous γ -secretase activity.

To address the role of PE in APP processing in *Drosophila in vivo* system, γ -reporter flies were crossed with **eas^{PC80f}** mutants, for the purpose of generating low PE flies expressing hAPP-Ct. Eas^{PC80} mutants have a defect in enzymatic activity of ethanolamine kinase (ETNK), which is the first enzyme in the synthesis of PE via CDP-ethanolamine pathway (Kennedy, 1957; Figure 1.4.). ETNK has been located on the gene *easily shocked* (*eas*) (Pavlidis et al., 1994), localized on the X chromosome. Eas^{PC80} mutation is a frame shift mutation based on a 2bp deletion in *eas^{PC80}* gene at nucleotide position 1004-1005, which introduces a stop codon in-frame at nucleotide position 1078. These mutants, defective in *eas* gene lack completely highly conserved kinase domain, which is required for ETNK enzymatic activity. Correspondingly to ETNK loss of function, eas^{PC80} mutants have a decreased PE levels compared to the wild-type flies. Eas^{PC80} homozygous are viable and under most conditions the flies do not show any observable abnormalities, regarding development, viability, behaviour and electrophysiology (Pavlidis et al., 1994).

In order to prove the effect of membrane PE on APP processing by γ -secretase in *Drosophila in vivo* system, eas^{PC80f} mutants were crossed with GMR-APP-GAL4, UAS-GRIM transgenic flies. At first, membrane PE levels were determined in eas^{PC80f} homozygous versus wild-type flies. Additionally, PE levels in the transgenic flies, which served as control for γ -activity levels, were measured in comparison to wild-type flies. TLC analysis of membrane lipid composition showed that eas^{PC80f} mutants have about 50% lower membrane PE levels than the wild-type flies. Ergosterol, ceramide, PECeramide, PC and PI membrane levels, in contrary, are not significantly different in mutant and wild-type flies (Figure 3.2.6. A). Transgenic and wild-type flies have a consistent membrane lipid composition, as shown in the Figure 3.2.6. B.

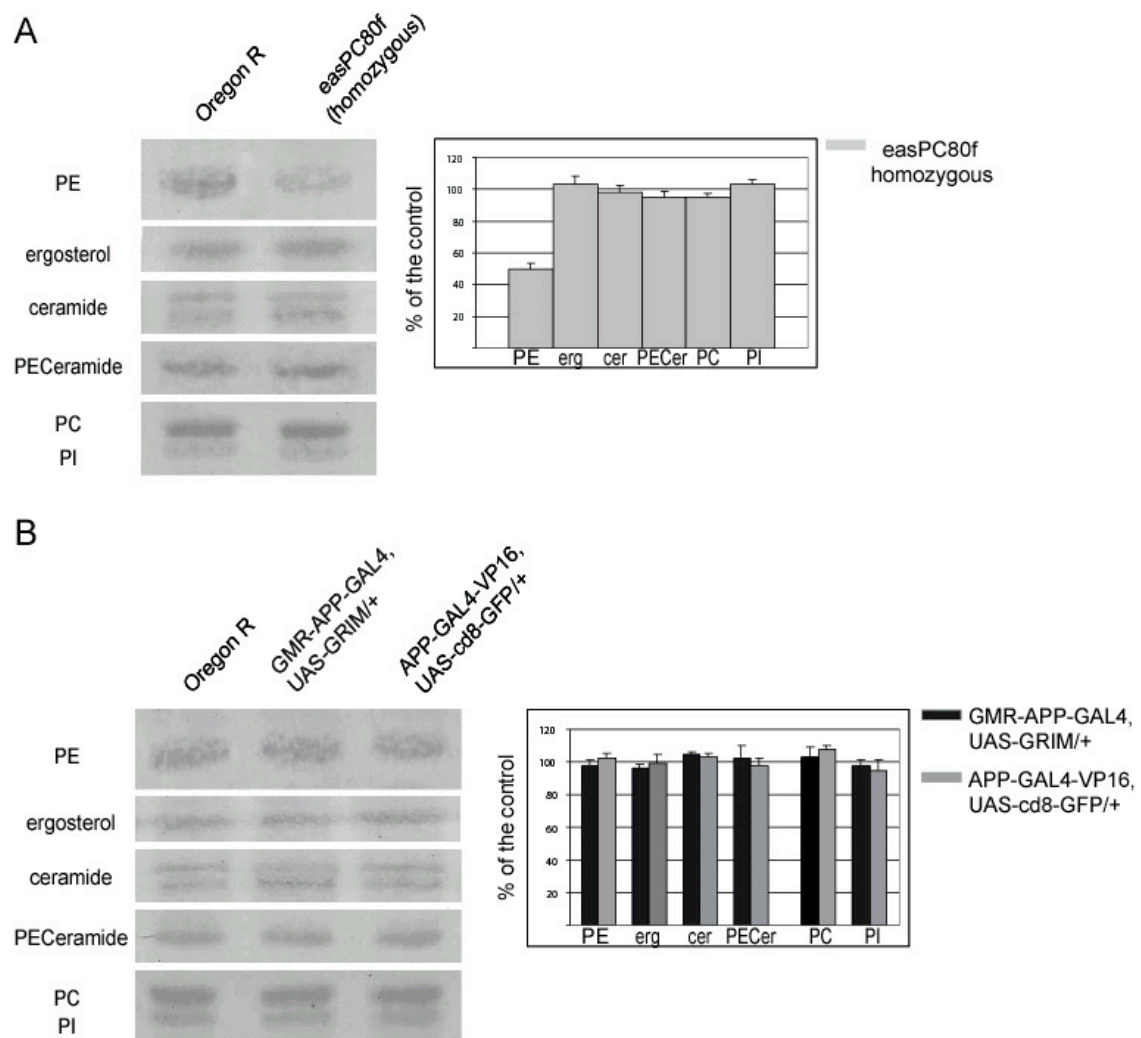
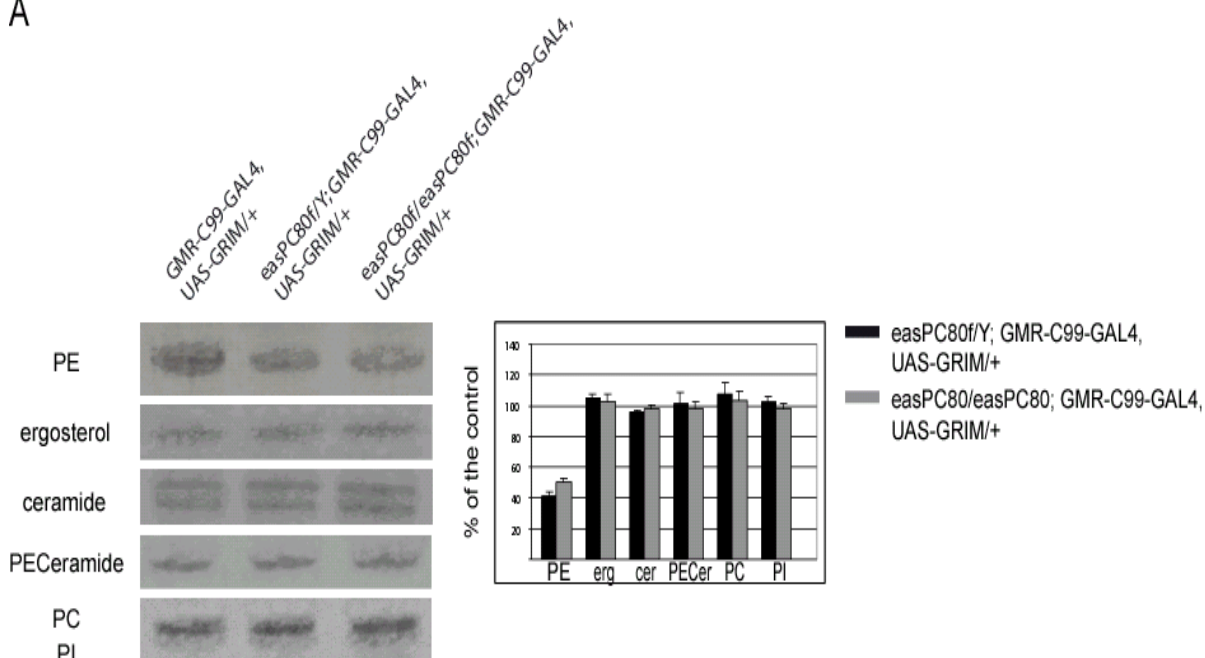


Figure 3.2.6. Eas^{PC80f} homozygous flies show a low membrane PE level versus wild-type flies, while membrane PE levels in γ -reporter flies do not differ from these in wild-type flies. Membrane pellets from homogenates of 10 flies were isolated by 100,000 $\times g$ centrifugation, using the same amount of total protein as starting material. Afterwards membrane lipids were extracted and analysed by TLC. **(A)** TLC analysis of membrane lipid content in eas^{PC80f} homozygous, lacking ETNK enzyme activity, revealed about 50% lower PE level in the membranes of these flies in comparison to the wild-type (Oregon R) flies. Mutants and control (wild-type) flies did not significantly differ in their membrane content of ergosterol, ceramide, PECeramide, PC and PI. Quantifications are represented in the graph. **(B)** Membrane lipid content in γ -reporter flies: GMR-APP-GAL4, UAS-GRIM/+ and APP-GAL4-VP16, UAS-cd8-GFP/+ versus wild-type flies shows similar values for PE, ergosterol, ceramide, PECeramide, PC and PI. For quantifications see graph.

Progeny, generated by crossing eas^{PC80f} mutants with GMR-APP-GAL4, UAS-GRIM transgenic flies with following genotype: **eas^{PC80f}/Y ; GMR-hAPP-Ct-GAL4, UAS-GRIM/+** and **eas^{PC80f}/eas^{PC80f} ; GMR-C99-GAL4, UAS-GRIM/+**, was assayed for membrane lipid content. Because hAPP-Ct is specifically expressed in the eye of the transgenic flies, membrane PE level in the head appears to be relevant for γ -secretase activity on APP. To determine membrane PE content, cell membranes were isolated by 100,000 x g from homogenates of 20 fly heads and analysed by TLC after lipid extraction. TLC analysis and subsequent quantification of membrane lipid content revealed a 50-60% lower membrane PE level in the progeny, which are γ -reporter flies homozygous for eas^{PC80} mutation, compared to the control flies, reporter flies with wild-type PE level. Other membrane lipids, like ergosterol, ceramide, PECeramide, PC and PI did not significantly alter in mutants and control flies (Figure 3.2.7. A).

A



B

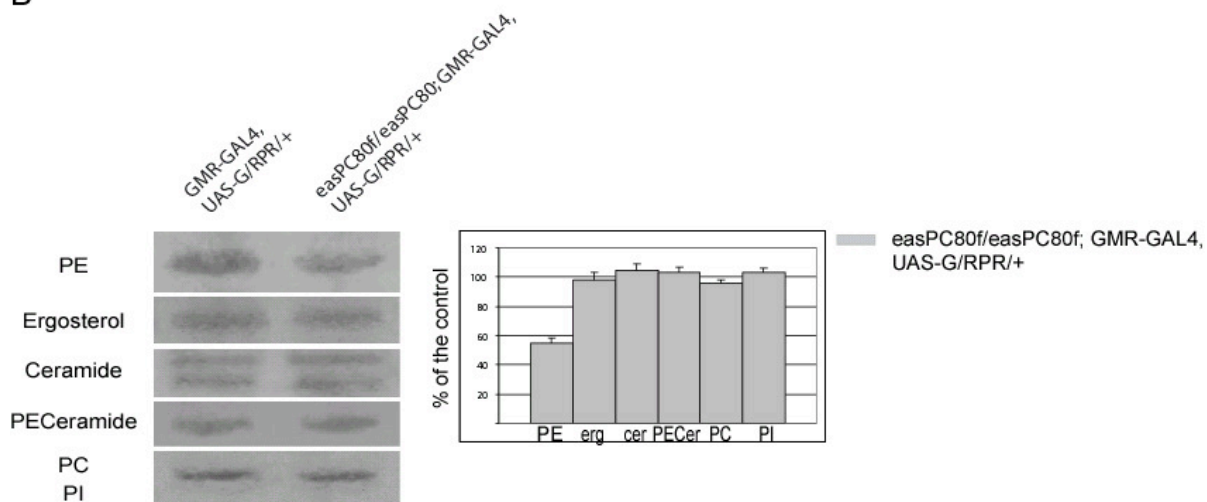
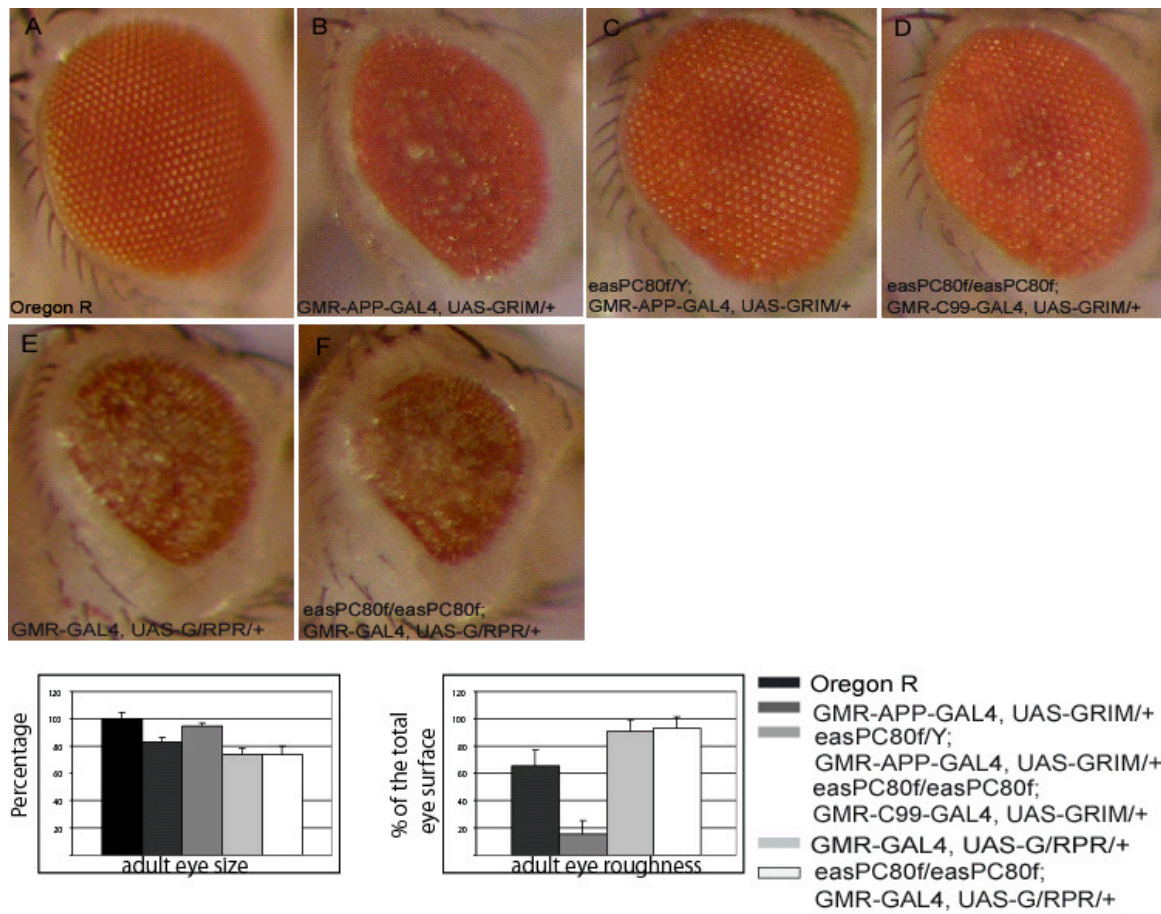


Figure 3.2.7. Membrane PE content is 50-60% lower in $eas^{PC80f/Y}$; GMR-hAPP-Ct-GAL4, UAS-GRIM/+ and $eas^{PC80f/eas^{PC80f}}$; GMR-hAPP-Ct-GAL4, UAS-GRIM/+ mutants, than in the transgenic flies without eas^{PC80} mutation, GMR-hAPP-Ct-GAL4, UAS-GRIM/+. (A) Membrane lipids extracted from the membranes of 20 fly heads were analysed by TLC and subsequently quantified for PE, ergosterol, ceramide, PECeramide, PC and PI amount. Membrane PE levels show a significant decrease of 50-60% in reporter flies carrying eas^{PC80} mutation compared to the control flies (GMR-hAPP-Ct-GAL4, UAS-GRIM). Other analysed lipids have similar membrane content in eas^{PC80} homozygous and control flies. (B) Membrane lipids isolated, under the same experimental conditions as described in A, have been assayed for their content in eas^{PC80} homozygous expressing GMR-GAL4, UAS-G/RPR/+ versus GMR-GAL4, UAS-G/RPR/+ flies without eas^{PC80} mutation (control). Membrane lipid quantification shows approximately 55% lower PE level in mutant flies with respect to the control flies. PE was only lipid, which shows altered levels in eas^{PC80} mutants, while ergosterol, ceramide, PECeramide, PC and PI contents do not significantly differ in comparison to the control flies. All quantifications are depicted in the respective graph.

These flies, which have low membrane PE level, relying on eas^{PC80} mutation and show retinal degeneration phenotype due to GMR-APP-Ct-GAL4, UAS-GRIM/+ transgene expression were analysed for the role of membrane PE in γ -activity. To assess the role of membrane PE in the modulation of γ -secretase activity,

eas^{PC80f} homozygous, carrying chimeric protein hAPP-Ct-GAL4-VP16 and the UAS-GRIM output were assayed for levels of retinal cell death versus control flies. Since *eas* gene is localized on the X chromosome, homozygous have been generated with *eas* mutation on one (eas^{PC80f}/Y ; GMR-hAPP-Ct-GAL4, UAS-GRIM/+) and on both alleles (eas^{PC80f}/eas^{PC80f} ; GMR-hAPP-Ct-GAL4, UAS-GRIM/+), to verify the results. Both homozygous have the same genetic background for ETNK activity and show corresponding membrane PE levels (Figure 3.2.7. A).

Effect of altered membrane PE levels on γ -secretase activity was analysed by the size of the fly eye and the size of roughened exterior eye surface. Both small eyes and roughened exterior eye surface are indicative for retinal cell death and correlate with the level of endogenous γ -secretase activity. Control flies, transgenic for GMR-hAPP-Ct-GAL4, UAS-GRIM with a wild-type membrane PE level (3.2.6. B and 3.2.7. A), show certain level of retinal cell death due to the normal levels of endogenous γ -secretase activity. Approximately 70% of total eye surface is roughened in the γ -reporter control flies and the eye size is compared to the wild-type eye about 20% decreased (Figure 3.2.8. A, B). Eas^{PC80f} homozygous, expressing GMR-hAPP-Ct-GAL4, UAS-GRIM were compared to the control flies measuring adult eye size and eye roughness, which was calculated as a percentage of the total eye surface. Retinal cell death, present in the control flies is almost eliminated and the eye size is restored to normal by introducing eas^{PC80f} mutation (Figure 3.2.8. B, C, D). Roughened eye surface in eas^{PC80f} homozygous averages only 10-15% of the total eye, compared to the 70% in control eye. Eye size in the flies with eas^{PC80} mutation is an average of 15% over the control, i.e. only 5% smaller than the wild-type eye, corresponding to decreased γ -secretase activity and less expression of the cell lethal gene.



G

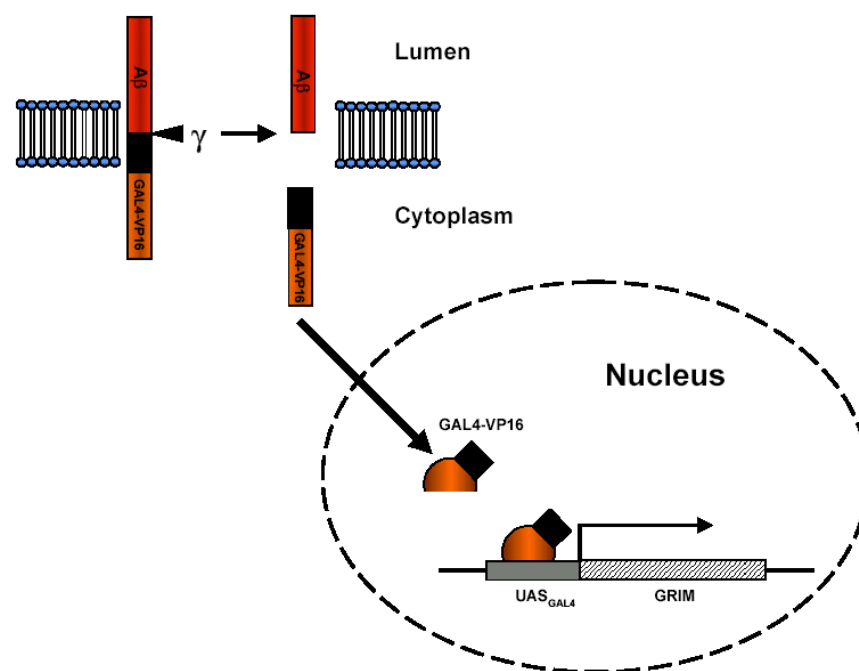


Figure 3.2.8. Levels of γ -secretase activity, measured by retinal cell death are significantly lower in reporter flies with eas^{PC80} mutation than in control γ -reporter flies. Light microscope images of adult fly eyes of various genotypes. The genotypes are as follows: **(A)** Oregon R (wild-type) **(B)** GMR-APP-GAL4, UAS-GRIM/+, **(C)** $eas^{PC80f/Y}$; GMR-APP-GAL4, UAS-GRIM/+ **(D)** $eas^{PC80f/eas^{PC80f}}$; GMR-hAPP-Ct-GAL4, UAS-GRIM/+. γ -secretase activity was measured by cell death levels in control reporter flies (GMR-hAPP-Ct-GAL4, UAS-GRIM; B) versus reporter flies homozygous for eas^{PC80} mutation (C, D). Retinal cell death was scored by quantification of eye size and the size of roughened eye surface. Quantifications of roughened eye surface revealed that the retinal cell death in mutant flies (C, D) was almost eliminated and so only 10-15% of eye surface were affected compared to 70% of roughened eye in control flies with wild-type levels of γ -activity. Also the eye size in eas^{PC80} homozygous mutants was similar to the one in Oregon R flies and approximately 15% bigger than in control flies. Measurement of retinal cell death in GMR-GAL4, UAS-G/RPR/+ control flies **(E)** versus eas^{PC80} mutants expressing GMR-GAL4, UAS-G/RPR/+ constructs (genotype: $eas^{PC80f/eas^{PC80f}}$; GMR-GAL4, UAS-G/RPR/+, **(F)**) revealed that the size of roughened eye surface and the eye size do not differ in mutants and control flies. Like in GMR-GAL4, UAS-G/RPR control flies eas^{PC80} mutants show approximately 90% of roughened eye surface. The eye size is 30% smaller than in the wild-type eye, in both mutant and control fly. **(G)** Schematic illustrating a γ -secretase reporter. The reporter contains two components: the chimeric protein (APP-CT (A β (red) + Ct (black bar)) appended to the GAL4-VP16) that serves as a γ -secretase substrate and the UAS-GRIM construct as an output. APP-GAL4-VP16 is specifically expressed in the eye under the control of GMR promoter. In the absence of γ -secretase activity APP-GAL4 remains attached to the membrane and is disabled to enter the nucleus and activate the transcription of the cell lethal gene GRIM. In the presence of γ -secretase activity, cleavage of APP releases a fragment consisting of APP-Ct initiating downstream of the γ -cleavage site and GAL4-VP16. This fragment translocates to the nucleus and activates GRIM transcription, thereby promoting retinal cell death through UAS activation.

To rule out an unspecific effect of eas^{PC80} mutation on GRIM expression, eas^{PC80f} mutants were crossed with the **GMR-GAL4, UAS-G/RPR** flies that do not contain hAPP-Ct chimeric protein. Like in γ -secretase reporter system, in control flies

GAL4-VP16 fusion construct is specifically expressed in the eye under GMR promoter and the flies show small eye phenotype. Since in control flies, GAL4 is not fused to hAPP-Ct, thus not under the control of γ -secretase, true modifiers of γ -secretase should alter the eye size of the reporter flies containing APP but not those of control flies, that lack APP. Flies carrying the *eas*^{PC80} mutation, which has been shown to correlate with altered γ -secretase activity, were crossed with control flies and analysed for membrane PE content and the level of retinal cell death.

Progeny, homozygous for *eas*^{PC80} mutation, containing GMR-GAL4, UAS-G/RPR constructs show about 55% decreased PE level compared to the control GMR-GAL4, UAS-G/RPR flies. Membrane levels of ergosterol, ceramide, PECeramide, PC and PI do not differ in mutant and control flies (Figure 3.2.7. B). Although *eas*^{PC80} mutants have a significantly altered membrane PE level, the comparison of retinal cell death in *eas*^{PC80} mutants versus control flies, revealed, that both eye size and the size of roughened eye surface are not significantly different (Figure 3.2.8. E, F). This observation indicates a specific effect of *eas*^{PC80} mutation through lowering of PE level, on γ -secretase activity.

To confirm the results obtained with GMR-APP-GAL4, UAS-GRIM transgenic system, **APP-GAL4-VP16, UAS-cd8-GFP** flies were utilized as transgenic readout to study γ -secretase activity. This system is based on the fusion of GAL4 DNA-binding domain and the VP16 activator domain to the C-terminal domain of hAPP (hAPP-Ct) (Figure 2.2.). Upon γ -secretase processing of hAPP-Ct, APP-Ct-GAL4-VP16 is released from the membrane, and it moves to the nucleus where it drives the expression of UAS-cd8-GFP reporter gene in a ligand-dependent manner (Struhl and Adachi, 2000) (Figure 3.2.10. C). Processing event of hAPP-Ct by γ -secretase is visualized by expression of the green fluorescent protein (GFP), which takes place in the whole body.

To study the modulatory role of PE on γ -secretase activity, PE levels in APP-GAL4-VP16, UAS-cd8-GFP flies have been modified, by crossing these flies with *eas*^{PC80} mutants. Subsequently GFP expression levels were determined in progeny homozygous for *eas*^{PC80} mutation. APP-GAL4-VP16, UAS-cd8-GFP

flies, which have wild-type PE levels (Figure 3.2.6. B), served as control for γ -activity. As described in the Figure 3.2.6. A, eas^{PC80} mutants show approximately 50% decrease in membrane PE content in comparison to the wild-type flies, whereas the content of other membrane lipids does not significantly differ in mutant and wild-type flies.

Before initiating analysis of γ -secretase activity, eas^{PC80} homozygous progeny generated by crossing APP-GAL4-VP16, UAS-cd8-GFP transgenic flies and eas^{PC80f} mutants were assayed for the membrane lipid composition. Cell membranes from homogenates of 10 fly bodies were prepared by centrifugation at 100,000 $\times g$. Subsequently membrane lipids were extracted and analysed by TLC (see Materials and Methods). TLC lipid analysis revealed that the level of membrane PE in progeny, homozygous for eas^{PC80} gene was 50% lower with respect to control flies. Levels of other membrane lipids: ergosterol, ceramide, PECeramide, PC and PI, did not significantly differ in the cell membranes of eas^{PC80} mutant and the control (Figure 3.2.9.).

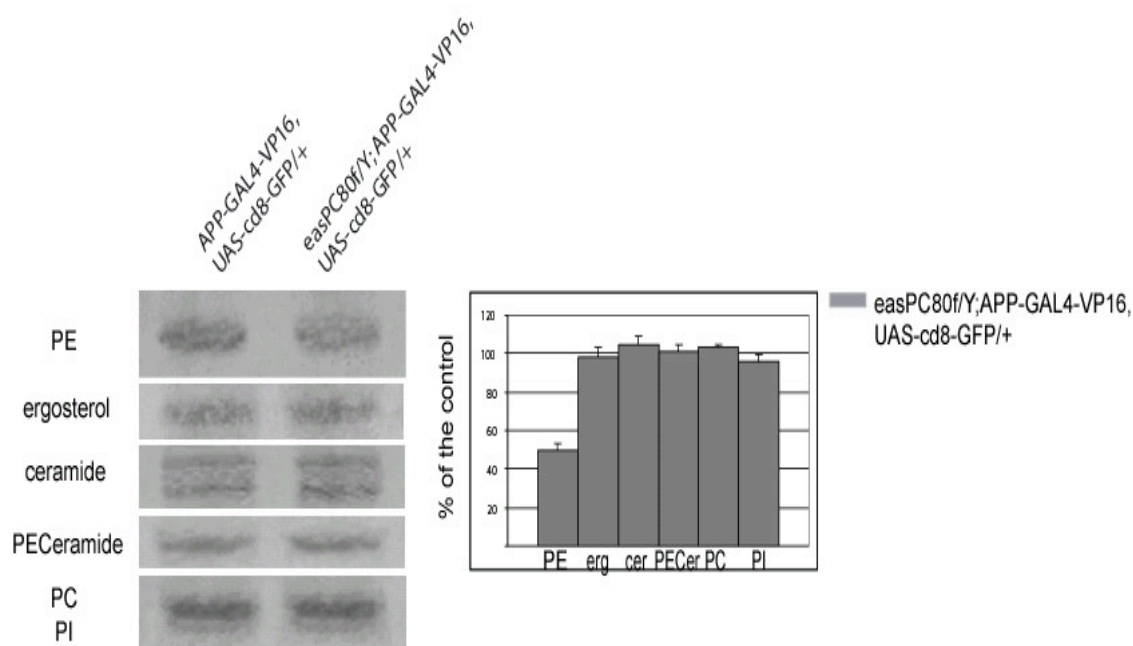


Figure 3.2.9. APP-GAL4-VP16, UAS-cd8-GFP/+ flies, homozygous for eas^{PC80} gene show decreased PE levels compared to the control flies. TLC analysis showing that only membrane PE levels reveal significant decrease of about 50% in eas^{PC80}

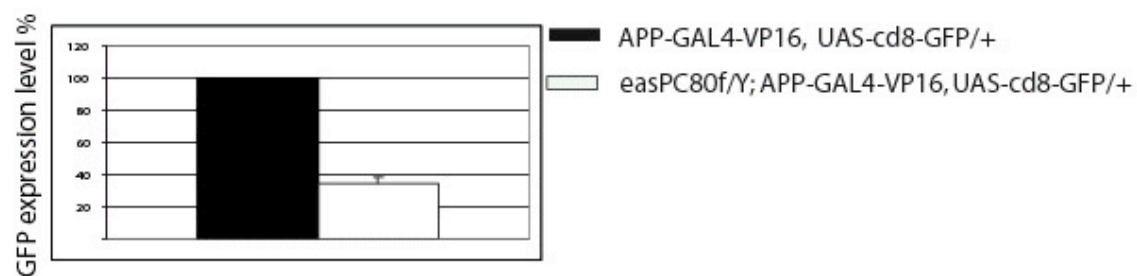
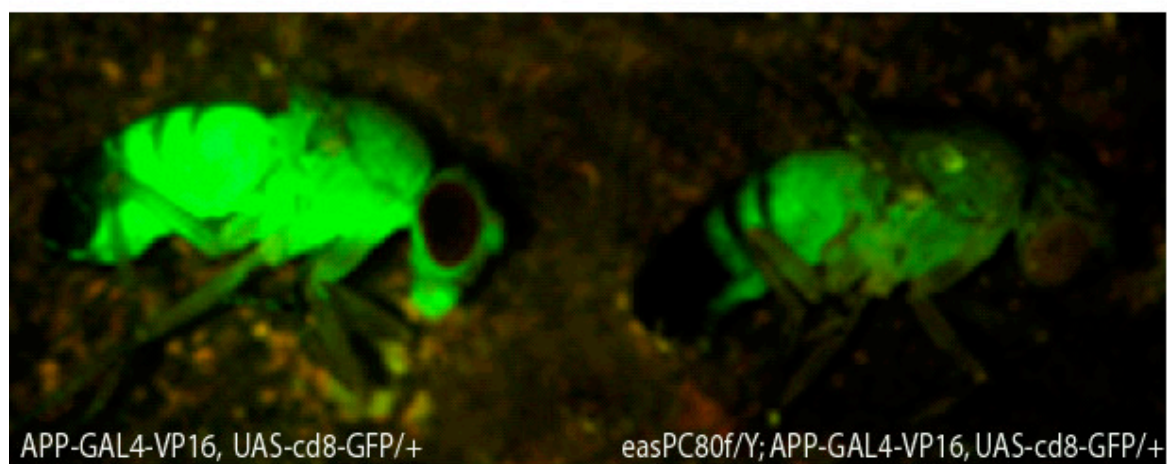
homozygous (eas^{PC80fY} ; APP-GAL4-VP16, UAS-cd8-GFP/+) compared to the control flies (APP-GAL4-VP16, UAS-cd8-GFP/+), whereas other lipids (ergosterol, ceramide, PECer, PC, PI) have similar membrane levels in both, mutant and control reporter flies. Values from membrane lipid quantification are depicted in the graph.

Whether PE depletion in the membranes of reporter flies, homozygous for eas^{PC80} gene, causes altered γ -secretase processing of hAPP-Ct was assayed by the GFP expression, measured by fluorescence intensity and Western blotting.

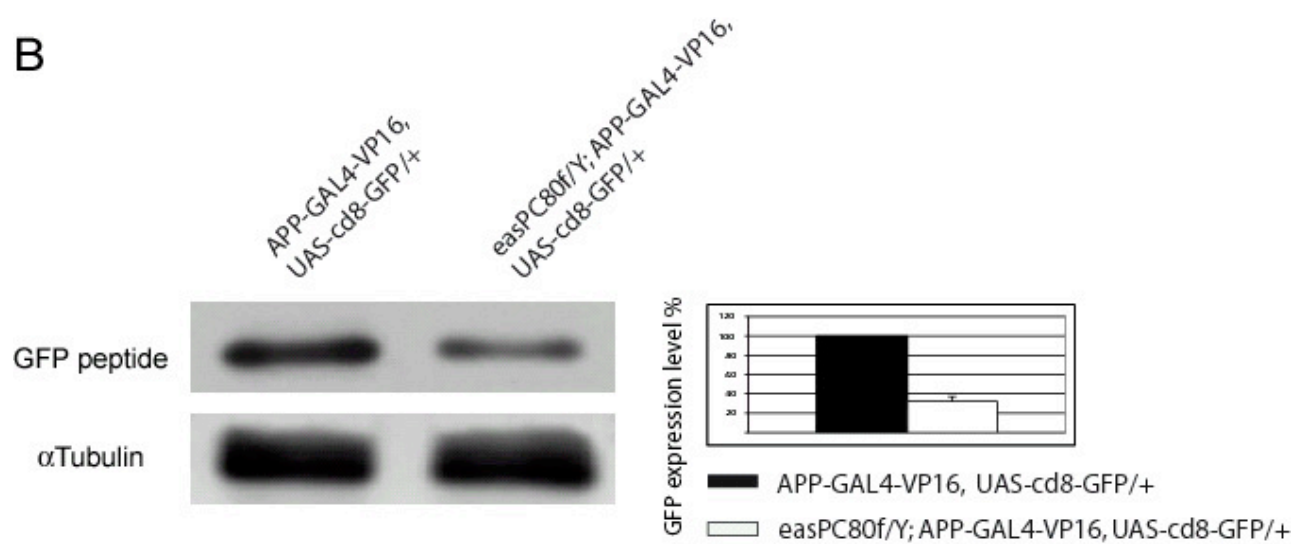
Transcription of GFP reporter gene is triggered by translocation of the C-terminal end of hAPP-Ct together with GAL4-VP16 to the nucleus after γ -secretase cleavage, so that GFP fluorescence level is a direct indicator of γ -secretase activity (Figure 3.2.10. C). In the absence or decrease of γ -activity, hAPP-Ct-GAL4-VP16 remains at the membrane and therefore is unable to enter the nucleus and activate GFP transcription. Analyses of GFP levels by fluorescence intensity and Western blotting were performed after adult flies were heat shocked at 37°C for 1 hour and placed at 25°C to allow GFP expression. Strong GFP fluorescence intensity, present in control reporter flies is an evidence for an active γ -secretase enzyme, under control conditions. After altering the membrane PE levels in reporter flies by introducing eas^{PC80} mutation, GFP levels decrease dramatically. Fluorescence intensity in GFP reporter flies with eas^{PC80} mutation is approximately 65% lower than in the control flies (Figure 3.2.10. A).

Additionally GFP levels in control and homozygous mutants were measured by Western blot analysis. 10 adult flies were homogenized in lysis buffer, subjected to 15% SDS page and stained with GFP-Peptide Antibody (BD Biosciences). As shown in the Figure 3.2.10. B, GFP expression, which corresponds to endogenous γ -secretase activity, is about 65% lower in mutant flies with respect to the control flies.

A



B



C

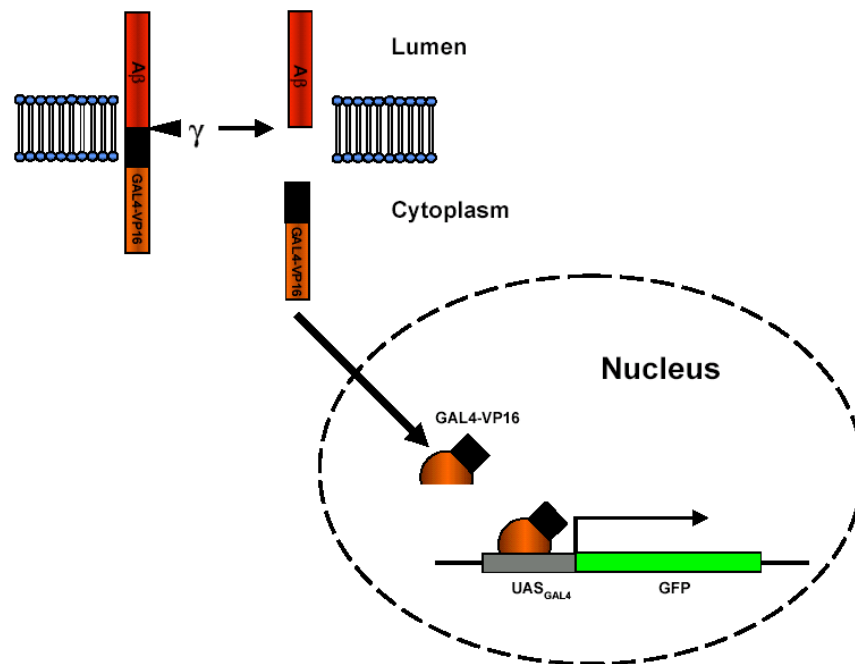


Figure 3.2.10. GFP expression in eas^{PC80} homozygous flies, transgene for APP-GAL4-VP16, UAS-cd8-GFP is dramatically decreased compared to the reporter control flies. (A) GFP signal in *Drosophila* transgenic flies with following genotypes: APP-GAL4-VP16, UAS-cd8-GFP/+ and eas^{PC80}/Y ; APP-GAL4-VP16, UAS-cd8-GFP/+, was measured 12h after heat-shock-induced activation of the APP-GAL4-VP16 construct. GFP expression measured by fluorescence intensity in the entire fly shows a decrease of about 65% in mutants with respect to control flies. (B) An additional analysis of GFP expression by Western blot analysis revealed, similar to the fluorescence intensity measurements, an approximately 65% lower GFP level in eas^{PC80} mutant versus control reporter flies. Tubulin served as the loading control. (C) Schematic illustrating a γ -secretase reporter. This system is based on a chimeric protein (APP-C-terminal fragment, consisting of: A β (red) + Ct (black bar)) appended to the transcription factor GAL4-VP16 (GV) downstream of APP-Ct transmembrane domain and the UAS-GFP construct as an output. In the absence of γ -secretase activity APP-GV remains tethered at the membrane and no GFP expression will be observed. γ -secretase activity releases APP-C-t-GV from the membrane that translocates to the nucleus and triggers GFP expression through UAS activation.

These data confirm the results obtained with the GMR-APP-GAL4, UAS-GRIM transgenic system and indicate a correlation of low membrane PE levels, caused by eas^{PC80} mutation, with the decreased level of γ -secretase activity in *Drosophila*.

4. Discussion

Present study shows that membrane PE levels correlate with alterations in APP processing. This finding is supported by the observation that APP localization is largely restricted to the phospholipid rich liquid disordered phase. Other than APP, the part of its cleaving machinery, involved in amyloidogenic pathway colocalizes with raft-like, liquid ordered membrane microdomains (Li et al., 2000, Wahrle et al., 2002, Ehehalt et al., 2003, Rodriguez et al., 2004, Vetrivel et al., 2004) though it is also clear that it is also present in the non-raft regions of the membrane. In contrast to β - and γ -secretase, α -secretases have not been linked to raft microdomains (Ehehalt et al., 2003). Given these facts APP proteolysis appears to be regulated by differential partitioning into distinct membrane sub-domains, rafts and non-rafts. In theory, such spatial segregation could determine the degree of access of secretases to the substrate and thus the degree of amyloid generation.

A key to understanding amyloidogenesis is to establish how the access of the secretases to APP is modulated. In this study I could demonstrate, by using *Drosophila* and mammalian model system, that the non-raft phospholipid rich membrane domains are implicated in processing of amyloidogenic APP. By no means this comes to contradict previous work strongly indicative of the involvement of rafts (Ehehalt et al, 2003); simply that non-rafts do also participate. From here one could conclude that APP proteolytic processing relies on dynamic interaction of raft and non raft membrane domains and that both rafts and non-rafts are important regulators of its cleavage by α -, β - and γ -secretase.

4.1. In mammalian HEK 293 cells membrane PE modulates proteolytic processing of APP by its cleaving enzymes α -, β - and γ -secretase

In the cellular membranes of HEK 293 cells, APP is mainly restricted to the phospholipid membrane core, which strongly indicates the involvement of these lipids in the regulation of APP cleavage. Indeed the analysis of APP processing

enzymes revealed significant changes in their activity in low membrane PE cells compared to the cells with wild-type membrane PE content.

For the purpose of analysing the role of PE in APP proteolysis, membrane PE levels were depleted by RNAi, directed against PECT, CEPT and ETNK enzymes that are involved in PE synthesis pathway. Inhibition of all three enzymes led to depletion of similar amounts of membrane PE. Moreover alterations in APP processing by its cleaving enzymes did not differ dependently on inhibited PE synthesis enzyme. These findings indicate that changes in APP cleavage observed in RNAi treated cells are not due to the inhibited enzyme activity itself, but to altered PE level caused by enzyme activity inhibition. Analysis of APP processing enzymes involved in amyloidogenic pathway show alterations in their activity in correlation with low membrane PE levels. The activities of γ - and β - secretases, read out by $A\beta$ and β CTF generation, respectively were significantly decreased after membrane PE depletion. In contrast, non-amyloidogenic α -secretase processing of APP, assayed by α CTF and sAPP α levels, revealed an elevated level in low PE cells. Composition of other membrane lipids, assayed in the membranes of RNAi treated cells, revealed that they do not significantly differ in comparison to the non-treated cells, so that the effect on the secretases activity seems to rely exclusively on the changes in membrane PE levels. Furthermore an unspecific effect of RNAi itself on APP cleavage by α -, β - and γ -secretase was excluded by treating the cells with RNAi directed against an irrelevant messenger RNA.

Regarding the finding above membrane PE appears to have benefit effect on the amyloidogenic pathway of APP processing, in contrast to the non-amyloidogenic APP processing, which is favoured in low membrane PE cells.

4.2. PE is implicated in the regulation of hAPP processing by γ -secretase in *Drosophila in vivo* system

Results obtained in mammalian HEK 293 cells, that strongly indicate a modulatory role of PE in APP proteolytic processing are supported by

observations in *Drosophila in vivo* system. As mentioned above amyloidogenesis depends on the interaction of γ -, β - and α -secretase. But since APP processing by γ -secretase represents the crucial step in the generation of the neurotoxic A β peptide modulation of this event appears to be most interesting for the regulation of AD implicated amyloidogenesis.

By using *Drosophila in vivo* system, I could demonstrate that membrane PE levels influence the modulation of γ -secretase activity on APP. Like in mammalian system, APP in *Drosophila* membranes colocalizes mainly with the phospholipid rich/cholesterol poor non-raft membrane domains.

In order to address the role of membrane PE in APP cleavage by γ -secretase, I analysed γ -activity in two different transgenic systems homozygous for *eas*^{PC80f} mutation. Low PE flies expressing C-terminal fragment of hAPP (hAPP-Ct) were obtained by crossing transgenic lines, carrying hAPP-Ct (**GMR-APP-GAL4, UAS-GRIM/+** and **APP-GAL4-VP16, UAS-cd8-GFP/+**) with low PE mutant flies (*eas*^{PC80f}).

Highly conserved γ -secretase activity in *Drosophila*, described by Iwatsubo and colleagues (Takasugi et al., 2002), has been shown in previous studies to processes hAPP in similar manner like in mammals (Fossgreen et al., 1998, Lichtenthaler et al., 1999, Struhl and Adachi, 2000, Loewer et al., 2003, Guo et al., 2003, Doglio et al., 2006). GMR-APP-GAL4, UAS-GRIM transgenic flies act as a sensitive and specific reporter of the endogenous, physiological levels of γ -secretase (Guo et al, 2003). Also APP-GAL4-VP16, UAS-cd8-GFP is an established model for γ -secretase analysis (Struhl and Adachi, 2000). As *eas*^{PC80} is a mutation with a known biochemical effect in phospholipid metabolism, particularly in PE synthesis (Pavlidis et al., 1994, Nyako et al., 2001) it allows the investigation of specific consequences of defects in phospholipids, or rather PE metabolism.

In GMR-APP-GAL4, UAS-GRIM reporter system hAPP-Ct-GAL4 is specifically expressed in the developing eye, in which the cell population is dominated by neurons. γ -secretase dependent cleavage of hAPP-Ct triggers the transcriptional activation of a cell death activator Grim, whose expression induces retinal cell

death. Thus, endogenous levels of γ -secretase activity correlate with the extent of retinal cell death, indicated by the eye size and the size of roughened eye surface. Control reporter flies with wild-type PE level show a small and roughened eye phenotype due to the wild-type levels of γ -secretase activity. A dramatic decrease of γ -activity indicated by almost eliminated roughened eye surface and to normal restored eye size, was obtained after reducing membrane PE content by *eas*^{PC80} mutation. An unspecific effect of *eas*^{PC80} mutation on retinal cell death was excluded in control experiments, performed in GMR-GAL4, UAS-G/RPR/+ transgenic system. Since these transgenic flies do not express APP and so GRIM expression is not γ -secretase dependent, true modulators of γ -activity should not affect the level of retinal cell death. In fact, GMR-GAL4, UAS-G/RPR/+ flies, homozygous for *eas*^{PC80} gene show a significant decrease in membrane PE content, but the level of retinal cell death do not differ from the GMR-GAL4, UAS-G/RPR/+ flies with wild-type PE amount.

Results obtained by using GMR-APP-GAL4, UAS-GRIM flies were confirmed in APP-GAL4-VP16, UAS-cd8-GFP transgenic system. In these flies endogenous γ -secretase activity is read out as levels of green fluorescent protein (GFP) expression. APP-Ct-GV translocates to the nucleus after being released from the membrane by γ -cleavage of APP, and leads to transcriptional activation of GFP protein, which is expressed in the whole fly. GFP expression determined by biochemical assay and fluorescence intensity measurements appears to be strongly decreased in transgenic flies homozygous for *eas*^{PC80} mutant gene with respect to the control flies. Decreased GFP expression in is an indicator for downregulated γ -activity in low PE flies, consistent to the results obtained in GMR-APP-GAL4, UAS-GRIM reporter system.

In both systems reporter gene transcription is triggered by translocation of GV and APP-Ct fragments, initiating downstream of the γ -secretase cleavage site, to the nucleus after γ -cleavage. As A β peptide is the counterpart of this γ -secretase generated APP-Ct fragment, a decrease of its generation complies with a decrease of A β peptide production.

As mentioned above eas^{PC80} is a mutation in ethanolamine kinase involved in PE synthesis via CDP-ethanolamine pathway, which has been shown to be the major source of PE in insects (Downer, 1985). Since ETNK is specifically involved in PE synthesis, the eas^{PC80} mutation is supposed to have no significant effect on membrane content of other lipids. Indeed, TLC analyses of *Drosophila* main membrane sterols, sphingolipids and phospholipids like ergosterol, ceramide, PECeramide, PC and PI revealed no significant alterations in their membrane content in eas^{PC80} homozygous with respect to control flies. Apart of a decrease in PE levels eas^{PC80} mutation does not alter membrane lipid content significantly, so that downregulated γ -secretase activity in eas^{PC80} homozygous appears to rely on low membrane PE levels. Although I cannot rule out the possibility of modulatory effect of the lipids which are not included in the analysis, it seems unlikely that one of those lipids would affect APP regulation in a such strong manner, considering their role in cell processes and their presence in the membrane in comparison to the major membrane lipids, e.g. PE or ergosterol.

4.3. PE depletion leads to downregulation of γ -secretase in neuronal and non-neuronal cells

Another very interesting aspect of the results obtained in GMR-APP-GAL4, UAS-GRIM and APP-GAL4-VP16, UAS-cd8-GFP flies is that PE affects γ -activity in a cell or tissue type independent manner. In GMR-APP-GAL4, UAS-GRIM transgenic system, γ -activity was analysed exclusively in neuronal tissue, whereas in APP-GAL4-VP16, UAS-cd8-GFP transgenic flies γ -secretase activity was assayed in whole fly, i.e. in different cell types. Given the results above PE effect on γ -secretase activity appears to be the same independent on cell or tissue type. Nevertheless PE modulation of γ -secretase in a cell type related manner cannot be excluded. It is possible that the modulations are manifested in more delicate manner, which was not observable in present systems.

Although present membrane PE decrease clearly induces downregulation of γ -activity in neuronal and non-neuronal *Drosophila* cells, it may be that the sensitive neuronal cells, different than other cell types, respond to very slight changes in PE membrane content.

Since PE appears to be involved in γ -secretase regulation, its membrane level modulation may be a target for therapeutic aims. For that purpose it would be interesting to determine the threshold of PE levels responsible for γ -alterations in neurons versus non-neuronal cells. Moreover this question remains to be answered because mild changes in membrane lipid content may be important for pathological situations. Neuronal tissue related processes, implicated in AD pathogenesis, are likely to be influenced by slight lipid alterations, as reported in mammalian system for the effect of moderate cholesterol loss on BACE1-activity and A β generation (Rodriguez et al., 2004).

4.4. PE effect on γ -activity differs in non-differentiated, embryonic *Drosophila* cells and in adult flies. Does PE correlate with γ -activity depending on cell differentiation stage?

An evidence for cell type related role of PE on γ -activity is provided by experiments done in embryonic *Drosophila* S2 cells. It is important to point out that S2 cells belong to embryonic cells, which are undifferentiated and so less complex than the cell types assayed for γ -activity *in vivo*. Different than in *Drosophila in vivo* system, membrane PE depletion in S2 cells induces a strong increase in γ -secretase activity.

S2 cells, transiently expressing hC99, were treated with RNAi directed against *eas* (ETNK) mRNA, encoded by the same gene that is mutated in *eas*^{PC80} flies. Additionally to membrane PE alterations by ethanolamine kinase (ETNK) RNAi, S2 cells were treated with RNAi against phosphoethanolamine cytidyltransferase (PECT). Inhibition of ETNK and PECT, both, enzymes involved in PE synthesis via CDP-ethanolamine pathway, causes PE depletion

comparable to the one in eas^{PC80} mutants. Like *in vivo* system, except of a significant PE decrease, the content of other membrane lipids was not affected by RNAi treatment. But other than *in vivo*, γ -secretase activity, measured by A β generation had clearly elevated levels in respect to the control cells with wild-type PE levels. A β content in the medium shows an enormous increase after PE depletion by RNAi treatment.

Although clearly dependent on membrane PE, alterations in levels of γ -secretase activity obtained in S2 cells do not correspond to those from mammalian HEK cells and *Drosophila in vivo* system. This discrepancy may be due to the embryonic, undifferentiated stage of S2 cells, that represents PE-mediated modulation of APP in a different way than mature, differentiated cells. This observation raises the question, whether PE modulation of γ -secretase activity depends on cellular differentiation and developmental stage.

Because the mechanisms responsible for A β generation are still poorly understood one may speculate that pathological accumulation of A β peptide in AD brains results from its perturbed generation at different developmental stages. Although the answer to this question may be interesting for understanding amyloidogenesis, the experimental approach provided by *in vivo* system appears more relevant for the aim of this study, as amyloidogenesis is an event, related to the differentiated adult cells.

4.5. Conclusion

Being an integral membrane protein APP is likely to respond to its lipid environment. Moreover APP proteolytic processing by α - β - and γ - secretase is a membrane occurring event. Hence, it seems likely that membrane lipids play an important role in the regulation of APP proteolysis. Findings presented in this study, show that in mammalian and in *Drosophila* system proteolytic cleavage of APP, which is largely restricted to the phospholipid membrane core, appears to be modulated by these lipids, particularly by PE. Even though the observations described here clearly indicate the PE involvement in APP proteolytic regulation,

it is important to point out, that neither PE alone, nor the phospholipid rich phase *per se* can account for full regulation of APP processing.

However, the way to understand the function of such a complex structure like cell membrane, which is composed of thousands of lipid species, is to analyze how individual lipids regulate activities of membrane proteins. In the present study I analysed the modulatory role of one of the major membrane phospholipids in APP proteolytic processing. I hypothesized that phospholipids play an important role in APP proteolysis, relying on the fact that APP mainly accumulates in phospholipid rich membrane core and I could establish that phospholipids, particularly PE modulates the processing of APP in the membranes of mammalian and *Drosophila* cells. As the experimental approach utilized in this study provides information on the mean role of PE in processing of APP the exact way by which PE regulates APP cleavage remains to be answered.

An explanation for the phospholipid modulatory role in APP proteolysis is the separation of the cleaving machinery and the substrate into distinct membrane microdomains. Growing evidence indicates the importance of rafts that are enriched in active β and γ -secretase, as principal membrane platforms for amyloidogenic processing of APP. As APP is mainly restricted to the non-raft domains analysis of rafts and non-raft interaction may provide essential answers about the regulation of APP proteolysis. It seems likely that phospholipid rich membrane sub-domains together with lipid rafts control the access of APP to its processing enzymes and that perturbed membrane phospholipid content disables the access of APP to the rafts, resulting in alterations of its proteolytic cleavage. This hypothesis is supported by findings obtained in the present study, which show that cleavage of APP by β - and γ -secretases, which are thought to be enriched in lipid rafts, decrease after PE depletion. Highly elevated levels of α -secretase products in low PE cells may be explained by the retention of APP in phospholipid rich domains caused by altered non-rafts properties. Under these circumstances APP accumulation in phospholipid rich membrane core would

provide an increased substrate amount for α -secretase, which is supposed to act in non-raft domains (Figure 4.1. A, B).

It is important to take in account that although active forms of γ - and β -secretase have been shown to enrich in lipid rafts, their activity is not exclusively restricted to these domains, i.e. γ - and β -activity on APP is present also in non-raft domains. Hence it is possible that γ - and β -secretase are trafficked from rafts to the non-rafts, to cleave APP. In this scenario altered non-rafts composition caused by depleted membrane PE would disable β - and γ -secretase to reach the substratum that would result in decreased amyloidogenic APP processing in non-raft domains (Figure 4.1. A,C), as shown in the present study. As mentioned above a downregulation in amyloidogenic processing increases the substrate amount for α -secretase, and consequently enhances the non-amyloidogenic pathway.

Whether APP needs to be transported to the rafts, where active γ - and β -secretases enrich or the processing enzymes are trafficked to their substratum to perform the cleavage remains to be answered. It is conceivable that these two events exist in parallel, i.e. that both rafts and non-rafts, provide the matrix for APP processing. However, the findings in the present study correspond to previous work, which suggests that the key event in the regulation of APP proteolysis is the restriction of enzyme accessibility to the substratum by partitioning them into distinct membrane microdomains, in rafts and non-rafts. In support to previous studies (reviewed in Kaether and Haass, 2004), which point out the role of cholesterol in APP cleavage, here I could show that PE, a major membrane phospholipid, that enriches in non-raft domains clearly influences APP proteolytic processing by α -, β -, and γ - secretase.

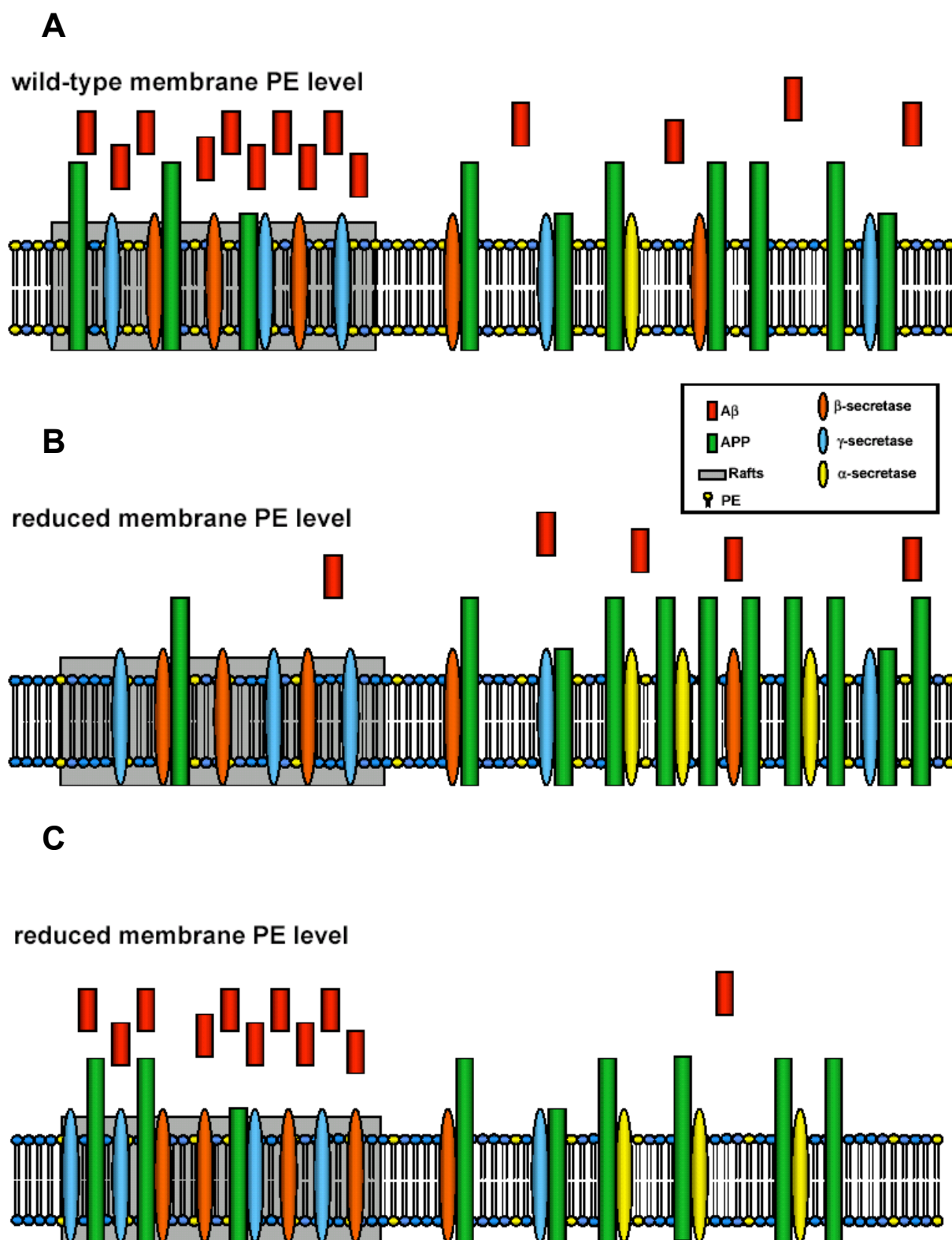


Figure 4.1. A model describing the effects of PE reduction on APP processing by secretases. For details see text.

Besides partitioning of the membrane proteins into distinct membrane microdomains, which is described above another way to restrict their contact is to separate them into different cellular compartments and dispatching them to separate trafficking routes. As this may be an important way in the modulation of APP processing by membrane lipids, the effect of membrane PE on APP in particular membrane compartments remains to be determined. More precise, it remains to be answered, whether membrane PE effect on secretases differs depending on their site of action, e.g. γ -secretase, which has been postulated to reside in multiple compartments including the ER, late Golgi/TGN, endosomes and plasma membrane (Cook et al., 1997, Xu et al., 1997, Greenfield et al., 1999, Takahashi et al., 2002 and Kaether et al., 2002), or β -secretase that has been reported to be the highest in late Golgi/TGN and endosomes (Koo and Squazzo, 1994, Haass et al., 1995, Walter et al., 2001) may respond more sensitive to membrane lipid alterations in these membrane compartments than in the others, in which the enzyme activity is supposed to be lower.

Another possibility, how PE may contribute to the regulation of APP proteolysis by secretases is by directly affecting the capacity of the enzyme to cleave the substratum. For instance, γ -secretase enzyme complex formation may depend on the proper organisation of phospholipid bilayer, so that perturbations in phospholipid rich domains caused by low membrane PE would not allow the assembly of an active γ -complex. Also α - and β -secretases are likely to depend on lipidic environment that provides the matrix for their activity.

I conclude that PE appears to have a benefit effect on APP cleavage by β - and γ -secretases, whereas low membrane PE levels enhance the processing of APP via non-amyloidogenic pathway. Since amyloidogenesis is a pivotal and early event in AD pathogenesis, membrane phospholipids may be important candidates for understanding the disease development and moreover for developing therapeutical agents for AD treatment.

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